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13. ABSTRACT *(Maximum 200 Words)*

Transforming growth factor-β (TGFβ) is a pleiotropic growth factor, which plays a critical role in modulating cell growth, differentiation and plasticity. There is increasing evidence that after cells lose their sensitivity to TGFβ-mediated growth inhibition, autocrine TGFβ signaling may potentially promote tumor progression. In order to further understand the role of TGFβ in mammary transformation, the MDA-MB-231 human metastatic breast cancer cell line was used to generate stable cell lines expressing a dominant negative TGFβ type II receptor (dnTβRII). Expression of dnTβRII was confirmed by affinity labeling cell surface receptors with ^{125}I-TGFβ1 and immunoprecipitating the affinity-labeled exogenous receptor via its HA tag. The dnTβRII was determined to be functional in that it could associate with the type I TGFβ receptor and prevent it from initiating signal transduction. Results to date indicate that the basal migratory potential of dnTβRII expressing cells is impaired, and this impairment appears to be TGFβ specific. Biochemical experiments are currently underway to identify the signal transducers perturbed by dnTβRII expression which impair motility, and which may, in turn, be relevant to TGFβ-mediated invasion and metastases.

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Table of Contents

Introduction

The overall goal of this research was to elucidate the complex role of transforming growth factor βs (TGFβs) in sequential stages of mammary epithelial transformation, and to determine whether blocking autocrine TGFβ signaling in MDA-231 human breast cancer cells would affect their metastatic phenotype *in vitro* and *in vivo*. This knowledge is of potential importance for the development of rational therapeutic approaches in human breast carcinoma.

Body

Specific Aim 1

The purpose of this Aim was to generate MMTV/tTA + tet-op/TGFβ1$^{S223/225}$ mice. As stated in our 1999 Annual report, although MMTV/tTA mRNA was expressed in the mammary glands of the F1 bigenic animals we generated, expression levels were very low, and tet-op/TGFβ1 mRNA was not detectable. Since this may have been due to the low level of expression of the MMTV/tTA, we obtained other MMTV/rtTA founder lines (1 heterozygous male and 1 heterozygous female) with more robust MMTV expression in the mammary gland from Dr. Lewis Chodosh (University of Pennslvania, PA). Unfortunately, the male MMTV/rtTA mouse died while being quarantined in our animal facility and the female MMTV/rtTA mouse died shortly after being released from quarantine. Consequently, breedings between these mice and our tet-op/TGFβ1 homozygous founder lines could not be set-up in order to generate the desired bigenic mice. We have since obtained two more male MMTV-rtTA mice from Dr. Chodosh. These mice are currently being quarantined in our mouse facilty. Once the two male MMTV-rtTA mice have been released from quarantine (scheduled for November 5, 2001), breeding pairs will be established between the MMTV-rtTA males and the tet-op/TGFβ1 females in order to generate the bigenic mice required for our studies.

Specific Aim 2

This Aim initially proposed to study the effect of mammary TGFβ1 overexpression on different stages of breast transformation in MMTV/neu + TGFα bigenic mice. Due to the complexity of generating a mouse between these two bigenics bearing four different transgenes, two alternative approaches were proposed in our 1999 Annual Report. However, until an appropriate MMTV/tTA + tet-op/TGFβ1 bigenic mouse is generated, these studies cannot be pursued.

Specific Aim 3

This Aim initially proposed to test the effect of antisense TGFβ1 and antisense TGFβ2 on MDA-231 human breast cancer cells. However, as stated in our 2000 Annual Report, since we were unable to generate stable transfectants with sustained expression of antisense, we chose to disrupt autocrine TGFβ signaling in these cells by stably expressing a dominant negative type II TGFβ receptor.

Generation of MDA-MB-231 Cells Stably Expressing a dnTβRII

To generate MDA-MB-231 cells stably expressing a dnTβRII, we obtained a construct encoding a kinase inactive TβRII in which the lysine at position 277 has been mutated to arginine (dnTβRII-K277R) (Oft et al., 1998) from Martin Oft (UCSF, San Francisco, CA). Lysine 277 corresponds

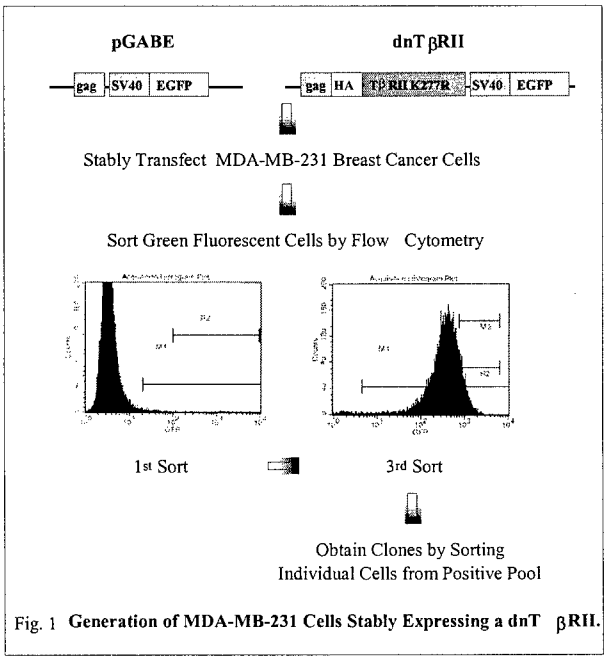

Fig. 1 **Generation of MDA-MB-231 Cells Stably Expressing a dnT βRII.**

to an invariant lysine found in the ATP-binding site of subdomain II in all protein kinases, and even its substitution with arginine results in loss of kinase activity (Wrana et al.,1992). The control pGABE and dnTβRII encoding vectors are modified versions of the commonly used retroviral vector pBABE in which the puromycin cassette has been replaced by enhanced green fluorescent protein (GFP). In this construct, dnTβRII-K277R is HA tagged, and its expression is driven by the viral long terminal repeat (LTR), while expression of GFP is driven by the SV40 promoter (Fig. 1). Following transfection, cells expressing GFP were sorted by flow cytometry. After multiple sortings, a pool of transfectants, the majority of which expressed GFP, was obtained (Fig. 1). Clones were then isolated by sorting individual cells from the positive pool.

Verification of dnTβRII-K277R Expression in MDA-MB-231 Cells

To verify that the dnTβRII-K277R mutant was indeed expressed in the pool and clones of MDA-MB-231 cells expressing EGFP, cell surface receptors were affinity labeled with ^{125}I-TGFβ1 (Fig. 2). As expected, cell surface labeling of parental cells resulted in the labeling of three proteins corresponding to the endogenous type I, II, and III TGFβ receptors expressed in these cells. There was little or no change in the amount of labeling observed in the control GABE clones. However, clones expressing dnTβRII-K277R, displayed a significant increase in the amount of labeled type II receptor, suggesting that the exogenous receptor was indeed expressed. A similar pattern of labeling was observed in the GABE pool from which the GABE clones were sorted, and the dnTβRII-K277R pool from which the dnTβRII-K277R clones were sorted.

In order to confirm that this increase in the labeling of the type II receptor was indeed due to expression of dnTβRII-K277R, extracts from affinity labeled cells were immunoprecipitated with an anti-HA antibody to pull down the HA-tagged dnTβRII-K277R. As shown in Fig. 3, the anti-HA antibody immunoprecipitated a labeled type II receptor in the pool and clones expressing dnTβRII-K277R, but not

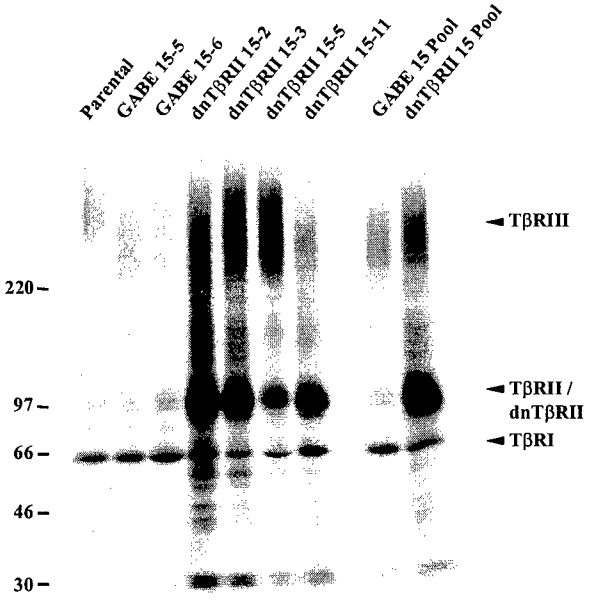

Fig. 2. **Expression of dnTβRII Increases Cell Surface Receptor Labeling**. MDA-MB-231 parental cells as well as clones and pools stably expressing dnTβRII or the control GABE vector were affinity labeled with 2.5 ng/ml ^{125}I-TGFβ1 and cross-linked with BS^3. Labeled ligand-receptor complexes were resolved by SDS-PAGE using a 3-12% gradient gel and visualized by autoradiography. Molecular weight markers are indicated on the left, and TGFβ receptors are indicated on the right.

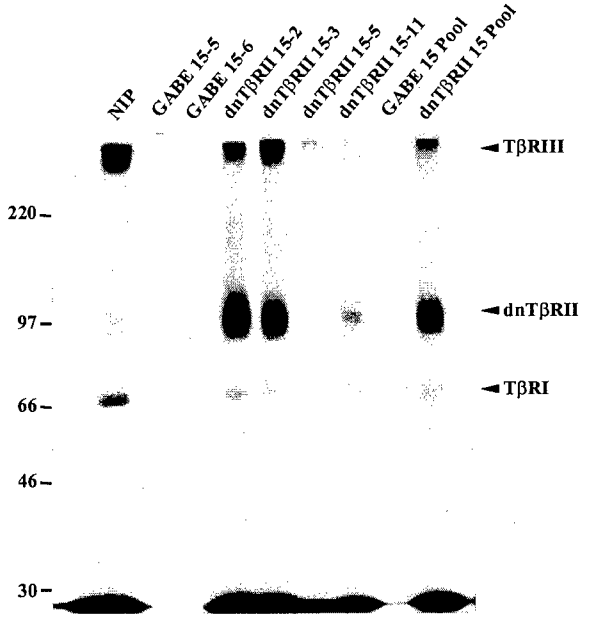

Fig. 3. **Immunoprecipitation of Affinity Labeled HA-Tagged dnTβRII.** Cells were affinity labeled with 2.5 ng/ml ^{125}I-TGFβ1, lysed and incubated with a mouse monoclonal anti-HA antibody for immunoprecipitation of HA-tagged dnTβRII. Immunoprecipitates were resolved by SDS-PAGE using a 7.5% polyacrylamide gel and visualized by autoradiography. Affinity labeled, but non-immunoprecipitated G15-5 cells were loaded as a reference (NIP).

in the control GABE pool or clones, confirming transgene expression. The type I and type III TGFβ receptors appeared to co-immunoprecipitate with dnTβRII-K277R in these experiments. This was confirmed in subsequent co-immunoprecipitation experiments (see Fig. 4 below).

Evaluation of dnTβRII-K277R Function in MDA-MB-231 Cells

Having ascertained that dnTβRII-K277R was indeed expressed in the MDA-MB-231 pool and clones, we then wished to determine whether it was functional. If so, it should associate with the type I receptor, prevent the endogenous type II receptor from associating with, phosphorylating, and activating the type I receptor, and therefore prevent the type I receptor from initiating signal transduction. The ability of dnTβRII-K277R to act as a dominant negative was evaluated utilizing four different assays. First, co-immunoprecipitation experiments were performed in order to determine whether dnTβRII-K277R could in fact associate with the type I TGFβ receptor (TβRI). Next, the effect of dnTβRII-K277R expression on various steps of TGFβ-mediated signal transduction was examined by i) immunoblot analysis of phospho-Smad2, ii) immunofluorescence staining to monitor Smad2 translocation to the nucleus, and iii) transcriptional activation of TGFβ-responsive reporter constructs.

i) Co-immunoprecipitation of TβRI with dnTβRII-K277R

The co-immunoprecipitation experiments revealed that when affinity labeled cells expressing dnTβRII-K277R were immunoprecipitated with an anti-HA antibody, a labeled protein the size of a type I receptor co-immunoprecipitated with the dnTβRII-K277R (Fig. 3 and Fig. 4, right panel – last lane). We confirmed that this was TβRI by immunoprecipitating similarly labeled cells with various TGFβ superfamily type I receptor antibodies, and observed that the only antibody which immunoprecipitated a type I receptor was the TβRI antibody, ALK5 (Fig. 4). These data suggest that dnTβRII-K277R can in fact associate with the type I TGFβ receptor.

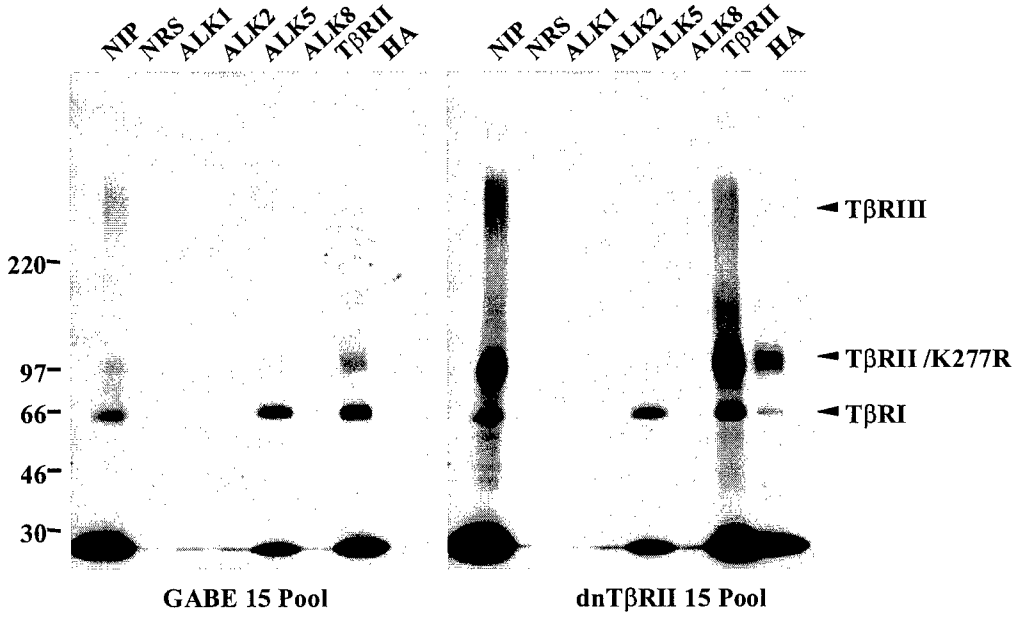

Fig. 4. **IP of ^{125}I-TGFβ1-Labeled Receptors.** MDA-MB-231 pools expressing dnTβRII (right panel), or control GABE vector (left panel) were affinity labeled with 2.5 ng/ml ^{125}I-TGFβ1, cross-linked with BS^3, lysed, and incubated with NRS (normal rabbit serum), polyclonal rabbit anti-sera directed against various type I receptors (ALK1,2,5,&8), the type II TGFβ receptor (TβRII), or HA, as indicated. Immunoprecipitates were resolved by SDS-PAGE using a 3-12% gradient gel and visualized by autoradiography. Affinity labeled, but non-immunoprecipitated cells were loaded as a reference (NIP).

ii) Impaired TGFβ-mediated Smad2 Phosphorylation in dnTβRII-K277R Clones

We next examined the effect of dnTβRII-K277R expression on TGFβ-induced Smad2 phosphorylation. Immunoblot analysis of TGFβ treated cell lysates using a phospho-specific Smad2 antibody revealed that while TGFβ could induce phosphorylation of Smad2 in both GABE clones (G15-5 and G15-6), its ability to do so in the dnTβRII-K277R clones was impaired (Fig. 5). This impairment was not due to a decrease in total Smad2 protein, as reprobing with an antibody directed against the unphosphorylated form of Smad2 did not reveal any significant change in total Smad2 protein.

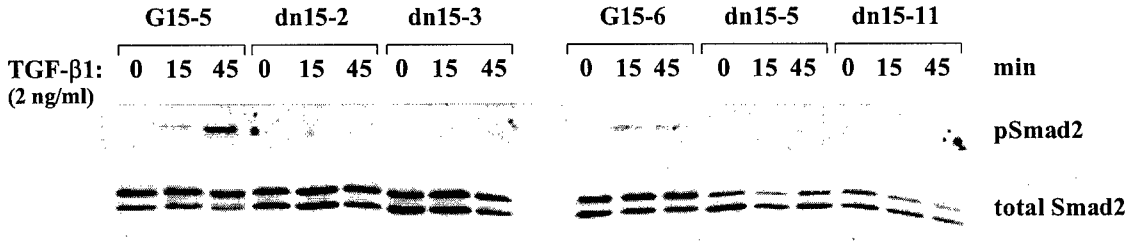

Fig. 5. **Impaired Smad2 Phosphorylation in dnTβRII Clones.** Cells were treated with 2 ng/ml TGFβ1 for the indicated times, washed, and lysed. Protein extracts (50ug/lane) were separated by 7.5% SDS-PAGE followed by immunoblot analysis for phospho-Smad2 and total Smad2.

iii) Impaired TGFβ-mediated Nuclear Translocation of Smad2 in dnTβRII-K277R Clones

Following phosphorylation, receptor-activated Smads associate with the common mediator Smad, Smad4, and translocate to the nucleus. Therefore, next we examined the effect of dnTβRII-K277R expression on TGFβ-induced Smad translocation to the nucleus by immunofluorescence (Fig. 6). In the GABE clone, in the absence of TGFβ, Smad2 staining was relatively diffuse, but upon TGFβ treatment for 60 min, Smad2 staining became concentrated in the nucleus. Nuclear localization of Smad2 was confirmed by staining the same cells with a nuclear dye. In contrast, in the dnTβRII-K277R clones, there was little or no change in Smad2 staining following TGFβ treatment, suggesting that TGFβ-mediated translocation of Smad2 to the nucleus is impaired by dnTβRII-K277R.

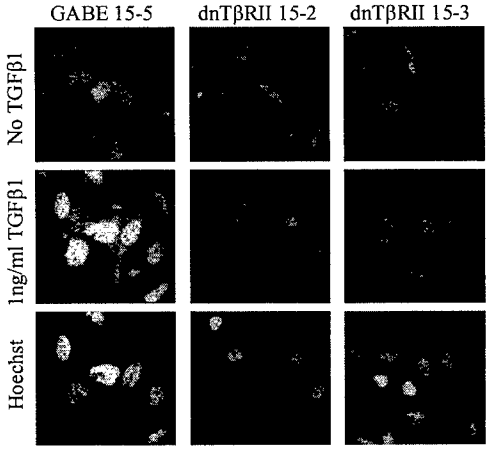

Fig. 6. **Impaired Smad2 Nuclear Translocation in dnTβRII Clones.** Cells were grown on glass cover-slips for 24h, serum starved for 16h, followed by treatment with 1 ng/ml TGFβ1 for 60 min. Cells were then fixed, permeabilized, and blocked with 3% milk in PBS for indirect immunofluorescence staining of Smad2 followed by nuclear staining with Hoechst. Cover-slips were mounted onto 25x75-mm microslides and fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axiophot upright microscope.

iv) Impaired TGFβ-mediated Transcription in dnTβRII-K277R Clones

Following translocation to the nucleus, Smads regulate gene transcription, so we examined the effect of dnTβRII-K277R expression on the ability of TGFβ to induce transcription of two TGFβ responsive reporter constructs, p3TP-luciferase, and pCAGA-luciferase. Each reporter construct was transiently transfected into the GABE and dnTβRII-K277R clones along with pCMV-Renilla, and luciferase activity before and after TGFβ treatment was measured utilizing a dual luciferase reporter assay system. Normalized luciferase activity indicated that TGFβ could induce transcription of both reporter constructs in the GABE clones (G15-5 and G15-6), but its ability to do so in the dnTβRII-K277R clones was impaired (Fig. 7).

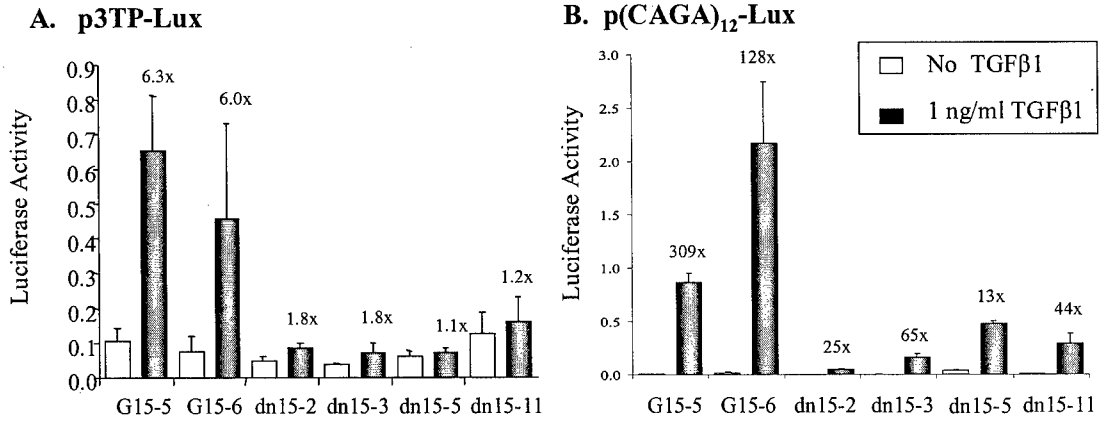

Fig. 7. **Impaired Transcription in dnTβRII Clones.** Cells were transiently tranfected with either **(A) p3TP-Lux** or **(B) p(CAGA)$_{12}$-Lux** (3ug/35mm dish) along with pCMV-Renilla (0.015ug/35mm dish). The following day, cells were split to 6 wells of a 24-well plate, treated with 1 ng/ml TGFβ1 for 16h, washed, and lysed. Firefly and *Renilla* luciferase activities were measured using Promega's Dual Luciferase Reporter Assay System. Luciferase activity (Y axis) is presented as the ratio of firefly to *Renilla* luciferase activities. Each data point represents the mean ± SD of three wells. Fold induction of luciferase activity for each clone is indicated above the bar representing luciferase activity following TGFβ1 treatment.

Taken together, these data indicate that dnTβRII-K277R is expressed and functional in MDA-MB-231 cells in that it can associate with TβRI (ALK5), and block every step of TGFβ-mediated signal transduction examined, including Smad2 phosphorylation, translocation of Smad2 to the nucleus and transcription. This system was therefore used as a model to study the molecular mechanisms by which TGFβ may be contributing to tumor maintenance and progression.

Although invasion and metastasis are complicated processes that involve multiple steps, active migration of cells into the surrounding tissue is a defining characteristic of the invasive tumor (Wells, 2000). Thus, as an *in vitro* biological assay that may be relevant to the metastatic phenotype *in vivo*, we chose to examine the effect of dnTβRII-K277R expression on the motility of MDA-MB-231cells in a wound closure assay. In this assay, a confluent monolayer of cells is wounded with a pipet tip, and the ability of the cells to migrate and close the wound is monitored by microscopy. In the GABE clones, complete wound closure was observed by 24h, while in the dnTβRII-K277R clones, although there was some closure, the wound remained open at 24h (Fig. 8). This difference in motility did not appear to be due to an effect on proliferation because when the experiment was performed in the presence of mitomycin C, a

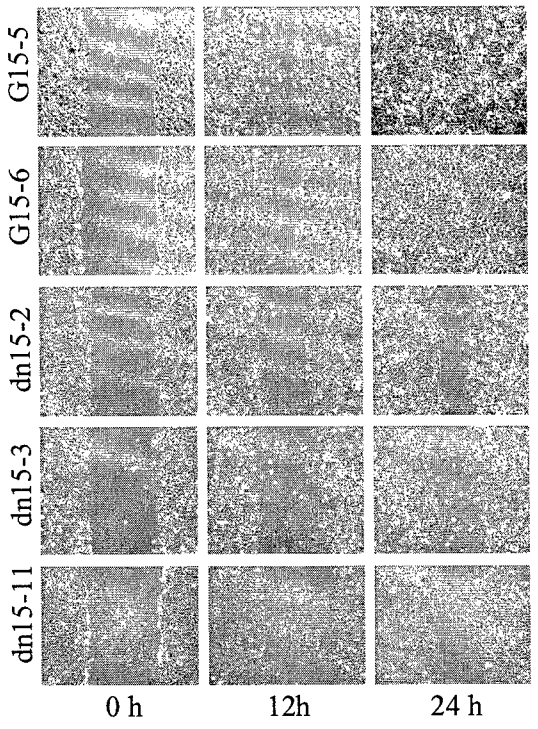

Fig. 8. **Impaired Motility in dnTβRII Clones.** Confluent cell monolayers were wounded with a pipet tip. Following wounding, cell culture medium was replaced with fresh medium containing serum and wound closure was monitored by microscopy at the times indicated.

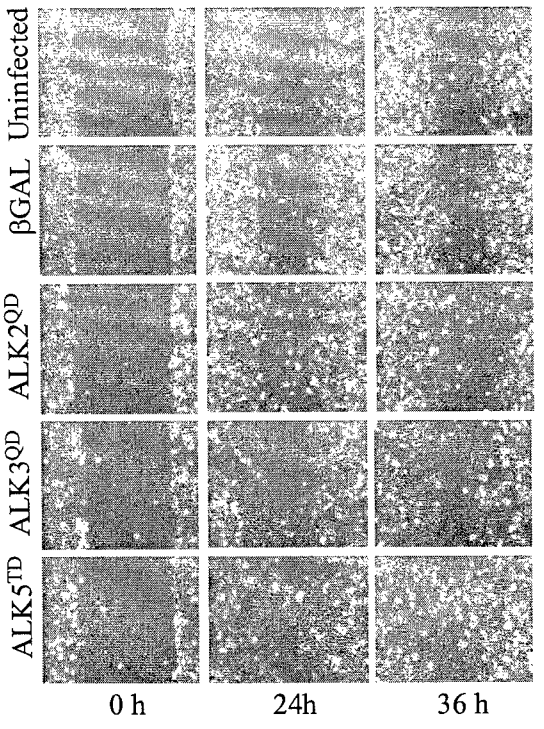

Fig 9. **ALK5 Restores Motility in dnTβRII 15-2.** Cells were infected with adenoviruses encoding constitutively active mutants of the BMP ($ALK2^{QD}$, $ALK3^{QD}$) or TGFβ ($ALK5^{TD}$) type I receptors. Uninfected cells, and cells infected with a β-GAL adenovirus were used as controls. Following infection, cell monolayers were wounded with a pipet tip, and wound closure was monitored by microscopy at the times indicated.

compound that inhibits cell division, the same results were obtained. Thus, expression of dnTβRII-K277R in MDA-MB-231 cells appears to impair their motility, independent of changes in proliferation.

Since we have data suggesting that in addition to blocking TGFβ signaling, expression of dnTβRII-K277R can also block BMP signaling in MDA-231 cells, we obtained adenoviruses encoding constitutively active mutants of TGFβ and BMP type I receptors in order to assess the relative contribution of each pathway in motility. Our results indicate that the impairment in motility observed in our cells appears to be TGFβ specific in that it could be restored by expression of a constitutively active type I TGFβ receptor ($ALK5^{TD}$), but not by constitutively active BMP type I receptors ($ALK2^{QD}$ and $ALK3^{QD}$), Fig. 9. Consistent with this, expression of Smad6, an inhibitory Smad which preferentially blocks BMP signaling, had no effect on the migration of parental or vector control cells in wound closure assays (Fig. 10).

Effect of dnTβRII-K277R Expression on MDA-231 Tumorigenesis and Metastasis

Tumor studies in nude mice were conducted in order to evaluate the effect of dnTβRII-K277R expression on tumor formation and metastases *in vivo*. However, these experiments were not informative as the control cells expressing GFP alone, failed to form tumors. We suspect that there may have been some toxicity associated with the expression of GFP as parental cells not expressing GFP did form tumors

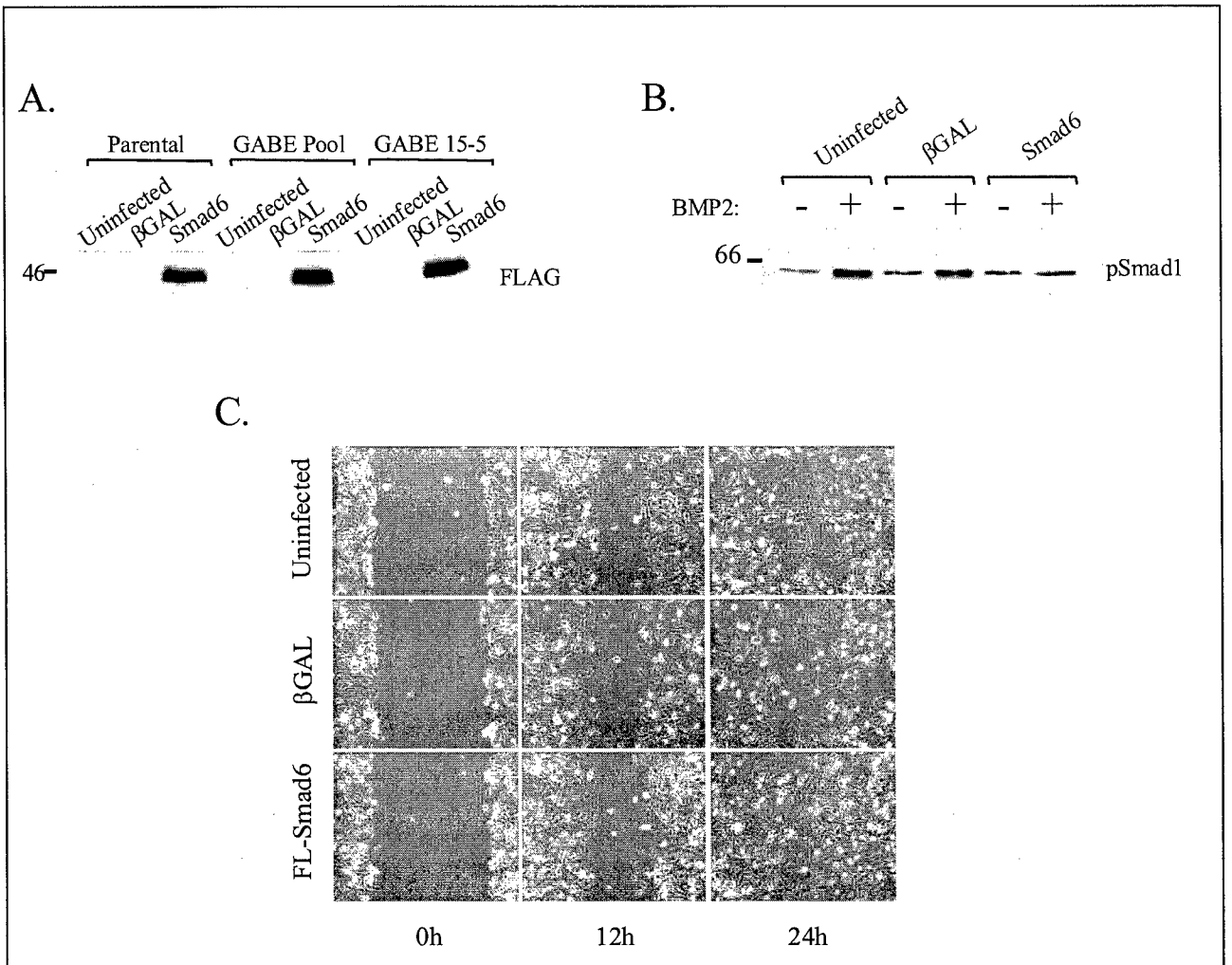

Fig. 10. **Smad6 Expression does not affect Motility**.
Cells were infected with Smad6 or control βGAL encoding adenoviruses. Following infection, Smad6 expression was verified by immunoblot analysis utilizing an anti-FLAG antibody **(A)**, and Smad6 function was evaluated by examining its ability to block BMP2-mediated Smad1 phosphorylation **(B)**. Despite being expressed and functional, Smad6 did not impair motility in wound closure assays **(C)**.

following subcutaneous or orthotopic injections in nude mice. We have therefore generated additional stable cell lines expressing dnTβRII-K277R without GFP, and are currently characterizing these for use in future *in vivo* studies.

Key Research Accomplishments

Generation and characterization of MDA-231 pools and clones stably expressing dnTβRII-K277R or control vector with or without GFP.

Reportable Outcomes

Manuscripts:

Dumont N. Genetic and epigenetic contributions to colorectal cancer. *APMIS* 107: 711-722, 1999.

Dumont N and Arteaga CL. Tumor promoting effects of transforming growth factor-β. *Breast Cancer Res* 2: 125-132, 2000.

McEarchern JA, Kobie JJ, Mack V, Wu RS, Meade-Tollin L, Arteaga CL, Dumont N, Besselsen D, Seftor E, Hendrix MJC, and Akporiaye ET. Invasion and metastasis of a mammary tumor involves TGF-β signaling. *Int. J. Cancer* 91: 76-82, 2001.

Abstracts:

Dumont N and Arteaga CL. Autocrine Transforming Growth Factor-β Signaling in Mammary Tumor Cell Invasiveness. Presented at the AACR Pathobiology of Cancer Workshop in Colorado, July 2000.

Dumont N, Bakin AV, and Arteaga CL. Expression of a Kinase Inactive TGFβ Type II Receptor Impairs Motility in Breast Cancer Cells. Presented at the Mammary Gland Gordon Conference in Rhode Island, June 2001.

Conclusions

1. The dnTβRII-K277R protein is expressed on the surface of MDA-231 cells, as evidenced by immunoprecipitation of ^{125}I-TGFβ1-labeled HA-tagged TβRII-K277R.

2. The dnTβRII-K277R is functional in that it can associate with the type I TGFβ receptor and impair various aspects of TGFβ signaling including:
   i) TGFβ-mediated Smad2 phosphorylation
   ii) TGFβ-mediated translocation of Smad2 to the nucleus
   iii) TGFβ-mediated transcription of TGFβ responsive reporter contructs (p3TP-luciferase and p(CAGA)$_{12}$-luciferase)

3. Expression of dnTβRII-K277R in MDA-231 cells impairs their motility.

4. The impairment in motility observed in cells expressing dnTβRII-K277R appears to be TGF-β specific in that:
   i) Motility can be restored by expression of a constitutively active TGFβ type I receptor, but not by constitutively active BMP type I receptors.
   ii) Motility is unaffected by expression of Smad6, an inhibitory Smad that preferentially blocks BMP signaling.

Since the dnTβRII-K277R is expressed and functional in MDA-MB-231 breast cancer cells, this provides an excellent model in which to study the molecular mechanisms by which TGFβ may contribute to tumor maintenance and progression. We have begun to do this utilizing migration as an *in vitro* biological assay that may provide insights into the mechanisms of invasion and metastasis. Biochemical experiments are currently underway to determine whether TGFβ-mediated motility is a Smad-dependent process that relies on changes in integrin expression and function. Identifying signal transducers involved in TGFβ-

mediated motility of breast cancer cells may provide important information for the selection of rational targets for therapeutic interventions. This may lead to opportunities to selectively inhibit the non-desirable tumor promoting effects of TGFβ while retaining its tumor suppressive effects.

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Wrana JL, Attisano L, Carcamo J, et al. (1992). TGF beta signals through a heteromeric protein kinase receptor complex. *Cell.* **71**:1003-14.

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Genetic and epigenetic contributions to colorectal cancer

Review article

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Dumont N. Genetic and epigenetic contributions to colorectal cancer. APMIS 1999;107:711–22.

Both genetic and epigenetic factors contribute to the development of colorectal cancer. Specific genetic changes in proto-oncogenes, tumor suppressor genes, and DNA mismatch repair genes have led to a genetic model of colorectal tumorigenesis. Recent data highlight the importance of the TGF-β signaling pathway in regulating the progression of colorectal cancer. The loss of the tumor suppressor activity of this pathway as well as the potentially cooperative genetic aberrations involving *APC*, *K-ras*, and *p53* are reviewed in the context of the multi-step adenoma-carcinoma sequence that characterizes the development of colorectal tumorigenesis. In addition, contributing epigenetic factors including age, diet, angiogenesis, and immune response are also discussed. Combining our knowledge of the genetic and epigenetic events implicated in this disease may allow a broader understanding of the pathogenesis of colorectal cancer and hence the design of better anti-tumor interventions.

Key words: Colorectal carcinoma; oncogene; tumor suppressor gene; mismatch repair; transforming growth factor-β; epigenetic.

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Like many cancers, colorectal cancer arises by acquisition of genetic alterations that result in cellular transformation. Based on these alterations, a genetic model for colorectal tumorigenesis has been proposed (1). However, in addition to genetic changes, several epigenetic factors have also been shown to contribute to the development of colorectal cancer. This paper reviews genetic as well as epigenetic contributions to colorectal cancer, both of which present opportunities as potential therapeutic targets.

GENETIC ABERRATIONS AND RELATED HISTOPATHOLOGICAL FEATURES

The development of colorectal cancer is a multistep process involving a series of genetic changes in the colonic mucosa that lead sequentially to hyperplasia, adenoma, carcinoma, and metastasis. Numerous genes, including proto-oncogenes, tumor suppressor genes, and DNA mismatch repair genes, have been implicated in the genesis of colon cancer. The discrete genetic changes currently perceived as fundamental to the multistep process of colorectal tumorigenesis are illustrated in Fig. 1. The tumorigenic process is initiated when a cell of the normal epithelium presumably undergoes a genetic change that conveys a selective growth advantage. This predisposes it to additional mutations, each of which confers further malignant potential, thereby leading to the clonal expansion of this cell (1). Thus, neoplasms of the colon are clonal in nature in that they arise from a single cell. Although sporadic mutations account for the majority of colorectal cancers, there are two hereditary syndromes in which a strong tendency to develop colorectal cancer is transmitted by dominant inheritance: Familial Adenomatous Polyposis (FAP) and Hereditary

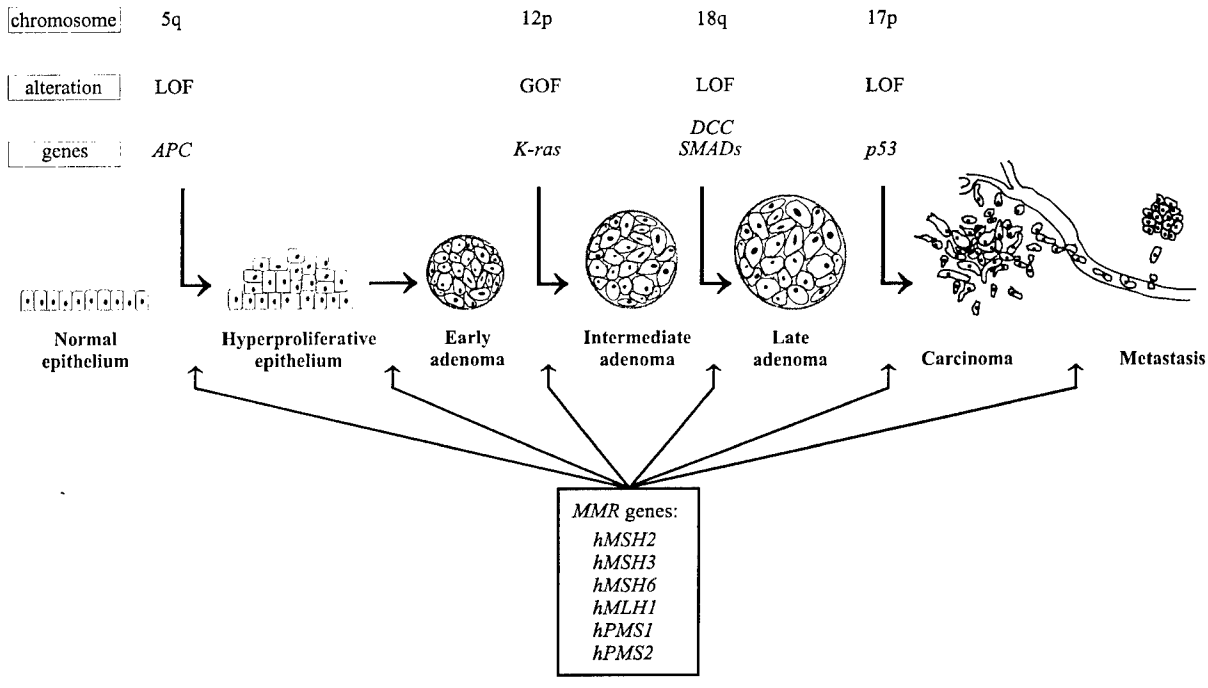


Fig. 1. Genetic changes associated with colorectal tumorigenesis. This process is accelerated by MMR deficiency (see text for details). Abbreviations: LOF, loss of function; GOF, gain of function; MMR, mismatch repair. Reproduced from Kinzler & Vogelstein (2) with modifications.

Nonpolyposis Colorectal Cancer (HNPCC) (2). FAP is a syndrome in which an inherited defect in the *adenomatous polyposis coli* (*APC*) gene leads to the development of multiple benign polyps throughout the colon, some of which slowly progress to invasive lesions. Thus, defects in *APC* initiate the tumorigenic process, but additional mutations, such as those illustrated in Fig. 1, are required for tumor progression. In contrast to FAP, HNPCC is a syndrome characterized by the rapid progression of colorectal tumors due to inherited defects in DNA mismatch repair (*MMR*) genes. Although the tumors from patients with HNPCC go through a series of mutations similar to those described in Fig. 1, additional mutations, unique to HNPCC, have been described. Evidence for the role of the genes most commonly implicated in sporadic and hereditary colorectal tumors are discussed individually below.

APC

One of the earliest steps in the development of colorectal cancer is loss of function of the tumor suppressor gene, *APC*. This gene was first identified as the gene responsible for FAP by demonstrating cosegregation of mutant *APC* alleles in affected kindreds (3, 4). However, the chromosome 5q region containing this gene is also frequently affected by loss of heterozygosity (LOH) events in colorectal adenomas and carcinomas from patients without polyposis (5–7). In fact, over 70% of sporadic colorectal cancers are believed to involve mutations in *APC* (8). Studies have shown that somatic mutations identified in sporadic tumors are similar to those observed in the germline of patients with FAP, often involving codon 1309 located in the mutation cluster region of the gene (9). These mutations have been identified in adenomas as small as 5 mm, consistent with the idea that mutations in the *APC* gene may be the initiating genetic event in many tumors (7). This is supported by the fact that excision of adenomatous polyps significantly reduces the incidence of cancer development (10). Further support for the role of *APC* in the development of polyps and colorectal cancer stems from studies of a mouse genetic model for FAP known as *Min* (for *m*ultiple *i*ntestinal *n*eoplasia). The *Min* mutation, like those in many FAP patients, causes

premature truncation of the APC protein, and mice heterozygous for the *Min* allele develop multiple adenomatous polyps and cancers in their intestine (11).

With respect to histopathological features, *APC* mutations lead to dysplastic lesions affecting the tube-shaped epithelial foldings of the colon referred to as crypts. Foci of dysplastic aberrant crypts are the earliest identifiable lesions in colorectal tumorigenesis, and are believed to be the precursors of adenomas (12). In FAP patients, different types of *APC* mutations are associated with different clinical features despite the fact that virtually all mutations result in C-terminally truncated APC proteins (2). These differences are manifested primarily in extracolonic sites, but may also be manifested by an attenuated form of FAP in which patients develop fewer polyps (13). This phenotype is also observed in the *Min* mouse where, depending on the inbred strain carrying the *Min* allele, wide variations in polyp number are seen. Linkage analysis has demonstrated that much of the variation is due to a single locus, named *MOM-1* (for *m*odifier of *M*in) (14), which encodes a secreted phospholipase A2 (15).

Although the exact mechanism by which *APC* mutations cause abnormal growth of colorectal epithelial cells is not clear, the fact that the majority of somatic and germline mutations in *APC* generate truncated APC proteins that lack a β-catenin-binding domain suggests that the interaction between APC and β-catenin may be important (16). Indeed, studies have shown that APC and glycogen synthase kinase 3 regulate cytoplasmic β-catenin levels by promoting its degradation (17). Inactivation of *APC* in colorectal cells allows β-catenin to accumulate and complex with T-cell factor 4, leading to activation of transcription and deregulated cell growth (18). The importance of APC and β-catenin in the development of colorectal cancer is further illustrated by the finding that β-catenin is mutated in a subset of colorectal cancers that lack somatic mutations in *APC* (19, 20). In addition to its role in regulating β-catenin, APC may also be involved in regulating apoptosis. Studies have shown that expression of wild-type APC in colorectal epithelial cells with *APC* mutations results in cell death (21). Thus, this may be another mechanism by which inactivation of *APC* leads to deregulated cell growth.

K-ras

Although the three *ras* genes, K-*ras*, H-*ras*, and N-*ras*, are highly homologous, and believed to be expressed at relatively equivalent levels in the colonic mucosa, only K-*ras* plays a significant role in the development of colorectal cancer. Mutations in K-*ras* can be identified in 50% of colorectal cancers (22), and occur most frequently in codon 12, with fewer mutations found at codons 13 and 61 (23). Because K-*ras* is an oncogene, mutation of one allele is enough to produce an effect. These mutations affect the ability of p21ras to interact with the ras GTPase-activating protein, causing p21ras to remain in the active GTP-bound state. As a result, the growth and differentiation signal transduction pathways that include p21ras are constitutively activated, leading to a continually growth-stimulated state.

The frequency of *ras* mutations appears to be correlated with two histopathological features: increased tumor size and dysplasia. Studies have shown that 50% of adenomas greater than 1 cm in diameter harbor K-*ras* mutations compared to only 10% of adenomas less than 1 cm (22). When adenomas are distinguished from one another with respect to the degree of dysplasia, *ras* mutations are more prevalent in tumors with increased dysplasia (24). The higher prevalence of *ras* mutations in later stage adenomas and carcinomas suggests that these mutations may arise in one cell of a small preexisting adenoma causing it to progress to a larger and more dysplastic adenoma, with greater risk of subsequent progression to cancer. This is consistent with the fact that hyperplastic cells containing mutant *ras* genes, unlike their dysplastic counterparts with mutant *APC* genes, have little or no potential to form clinically important tumors and may eventually regress through apoptosis (25).

DCC, Smad4, and the tumor suppressor activity of the TGF-β pathway

LOH affecting the long arm of chromosome 18 can be detected in more than 70% of primary colorectal cancers, in about 50% of advanced adenomas, and infrequently in earlier stage adenomas, suggesting that loss in this region is a relatively late event (22). Such losses are also correlated with greater mortality and increased propensity for metastatic spread (26, 27). Ef-

forts to identify a candidate tumor suppressor gene from 18q led to the discovery of a gene termed *DCC* (for *d*eleted in *c*olorectal *c*ancer) (28). The *DCC* gene encodes a transmembrane protein of the immunoglobulin superfamily. The predicted structural similarity of DCC to the N-CAM family of cell-surface adhesion molecules suggested that it might function in differentiation pathways and cell fate determination through cell-cell and/or cell-extracellular matrix interactions (29). Therefore, it was hypothesized that loss of cell-cell contact might explain the enhanced metastasis observed in patients with loss of *DCC* (29). However, more recent studies have shown that inactivation of the murine *DCC* gene does not affect the proliferation or differentiation of intestinal epithelial cells, nor does it affect the morphogenesis of colonic crypts and villi (30). Moreover, introduction of the null *DCC* allele into the germ line of the *Min* mouse does not accelerate the progression of, or modify the phenotype of polyps initiated in the *Min* mice (30). Instead, the phenotype of mice lacking a functional *DCC* gene resembles that of netrin-1-deficient mice, with defects in axonal projections and brain development (30). These findings fail to support a tumor suppressor function for *DCC* in the development of colorectal cancer, and are inconsistent with studies in which reduction or loss of *DCC* RNA has been observed in cell lines or xenografts derived from human colon carcinomas (28, 31).

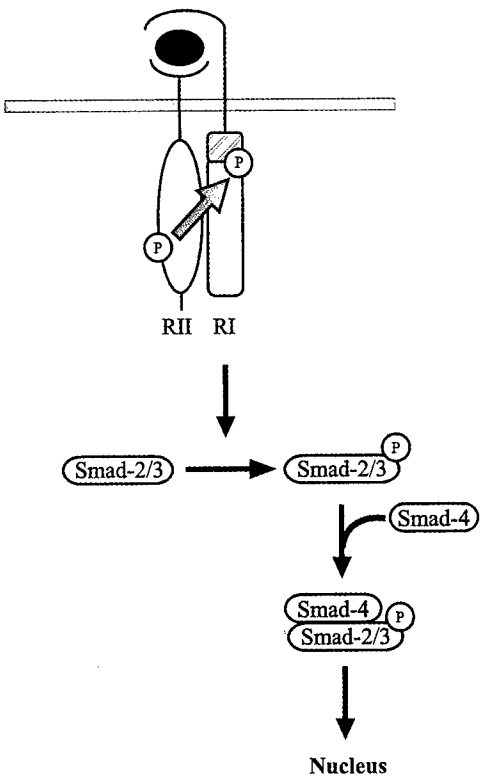
The discrepancy between these results may be due to differences in the pathogenesis of colorectal cancer between mice and humans. Alternatively, LOH of 18q21 may not only affect the *DCC* gene, but neighboring genes as well, one or more of which may be the target of inactivation during colon tumor progression (30). Indeed, other candidate tumor suppressor genes, including *Smad2* and *Smad4*, have been identified on chromosome 18q21 (32, 33). Both *Smad2* and *Smad4* belong to the *SMAD* gene family involved in the signal transduction pathways activated through the TGF-β family of receptors (34). The TGF-βs are important regulators of cell growth and differentiation (35). Escape from the growth regulatory effects of TGF-βs is common among many different cancers (35, 36), including colorectal cancer (37). Recent studies have shown that inactivation of the *Smad4* gene in $APC^{\Delta 716}$ knockout mice, which have a phenotype similar to that of the *Min* mice, results in malignant progression of the intestinal tumors at a much earlier stage than that observed in the simple $APC^{\Delta 716}$ knockout mice (38). Mice heterozygous only for the *Smad4* knockout show no apparent tumor phenotype, indicating that the *Smad4* gene is a suppressor of tumor progression, but not of tumor initiation (38). The fact that inactivation of *Smad4* in $APC^{\Delta 716}$ knockout mice enhances tumor progression, while inactivation of *DCC* in a similar mouse model does not, suggests that the tumor suppressor gene associated with LOH on 18q21 is more likely to be *Smad4*. In support of this, mutations in the *Smad4* gene have been identified in human colorectal cancers in vivo (33, 39, 40), and in familial juvenile polyposis, which, like FAP, is a syndrome characterized by a predisposition to hemartomatous polyps and gastrointestinal cancer (41).

A role for *Smad4* in the malignant progression of colorectal tumors is consistent with previous findings that have implicated other members of the TGF-β pathway in colorectal tumorigenesis (summarized in Fig. 2). For example, as previously mentioned, *Smad2* is a candidate tumor suppressor gene also located on 18q21. Inactivating missense mutations and deletions of the *Smad2* gene have been detected in sporadic colorectal carcinomas (32, 42). In addition to Smad2 and Smad4, Smad3 is another member of the SMAD family of proteins involved in mediating TGF-β signaling. Although mutations in the *Smad3* gene have not yet been detected in human colorectal cancer (43, 44), LOH of *Smad3* has been detected in 2 (1 sporadic and 1 HNPCC) of 17 colorectal cancers examined (44), and a recent study has reported that inactivation of the *Smad3* gene in mice leads to the development of metastatic colorectal cancer (45). Upstream of the SMAD signaling proteins, mutations in the TGF-β type II receptor (TGF-βRII) have been associated with microsatellite instability in both colon cancers and colorectal carcinoma cell lines (46–48). Restoration of TGF-βRII expression by gene transfection has been found to reverse the transformed phenotype (49). There is also evidence that transfection of TGF-β-responsive human colon carcinoma cells with a TGF-β1 antisense expression vector increases their tumorigenicity (50). These data taken together highlight the im-

portance of the TGF-β signaling pathway in regulating the progression of colorectal cancer.

p53

The chromosomal region most frequently affected by LOH in colorectal cancers is 17p (51), a region which includes the tumor suppressor gene, *p53*. Sequence analysis of the remaining *p53* allele from a large number of colorectal carcinomas in which 17p was affected by LOH revealed that missense mutations were present in over 80% of the cases (52, 53), indicating that loss of *p53* function is an important step in the development of colorectal cancer. Although *p53* mutations are extremely prevalent in many advanced colorectal cancers, they are much less frequent in earlier stages of carcinogenesis, and rare in both adenomas and polyps (22). Moreover, patients with germline mutations in *p53* do not develop polyposis, nor are they at risk for developing colorectal cancer (54). This suggests that, although *p53* plays an important role in colorectal carcinogenesis, unlike *APC* it cannot initiate the process. Likewise, *p53*-deficient mice are prone to develop cancers, but an initiating event is required for tumor development (55, 56). Moreover, the incidence of colorectal cancer in these animals is low, indicating that loss of *p53* function alone does not cause transformation (57). This is consistent with the role of p53 as a cell-cycle checkpoint regulator (58). Abrogation of either p53-



TGF-β signaling component	Role in colorectal tumorigenesis	Ref.
TGF-β	-Loss of sensitivity of colon cancer cells to growth inhibitory effects of TGF-β	37
	-Antisense TGF-β1 increases tumorigenicity of human colon cancer cells	50
TGF-βRII	-Mutated in colon cancers and in colorectal carcinoma cell lines with MSI	46-48
	-Restoration of RII expression reverses transformed phenotype	49
Smad2	-Mutated in sporadic colorectal cancer	32,42
Smad3	-*Smad3-/-* mice develop metastatic colorectal cancer	45
	-LOH in 2 (1 sporadic and 1 HNPCC) of 17 cancers examined	44
Smad4	-Inactivation of *Smad4* in $APC^{\Delta 716}$ mice enhances tumor progression	38
	-Mutated in human colorectal cancers *in vivo*	39-40
	-Mutated in familial juvenile polyposis	41

Fig. 2. The TGF-β signaling pathway and its role in colorectal tumorigenesis. TGF-β (represented as a circle) elicits its effects through binding to specific cell-surface receptors denoted type I (RI) and type II (RII) TGF-β receptors, both of which are transmembrane serine/threonine kinases. TGF-β binds directly to RII, which is a constitutively active kinase. The ligand-bound RII then recognizes RI, leading to the formation of a heteromeric complex, allowing RII to phosphorylate and thereby activate RI. The activated RI phosphorylates Smad-2 or Smad-3, which then associates with Smad-4. This complex translocates to the nucleus where it can initiate gene transcription. TGF-β, RII, Smad-2, Smad-3, and Smad-4 have all been implicated as possible tumor suppressors in colorectal cancer, as summarized in the table above (see text for details). Abbreviations: MSI, microsatellite instability; LOH, loss of heterozygosity. Signaling pathway reproduced with modifictions from Massague (34).

dependent cell-cycle arrest or apoptosis could allow cells that have incurred mutations and are not fit to progress through the cell cycle to do so anyway, resulting in tumor progression.

With respect to clinical features, the presence of mutant p53, as determined by increased immunohistochemical staining or by analysis of gene sequence, is correlated with poor survival, as well as increased cancer recurrence rates (59, 60).

MMR genes

DNA mismatch repair plays a prominent role in the correction of replicative mismatches which escape DNA polymerase proofreading. Three genes, *MutS*, *MutL*, and *MutH*, are central to the correction of replication errors in E. coli (61). In humans, germline mutations in the *MutS* homologue, *hMSH2* (62, 63), or in any of the three MutL homologues, *hMLH1*, *hPMS1*, and *hPMS2* (64–66), have been identified as being responsible for HNPCC. These reports suggest that mutations in *hMSH2* and *hMLH1* together account for the majority of HNPCC, while mutations in *hPMS1* and *hPMS2* are less frequently observed. A characteristic feature of tumors arising in individuals with HNPCC is the presence of microsatellite instability. Microsatellites are regions consisting of single, dinucleotide, or trinucleotide repeats that are widely distributed throughout the genome. These sequences are prone to replication errors due to their repetitive structure, which favors slippage during replication. Thus, when *MMR* genes are mutated, errors arising during DNA replication are less efficiently corrected, resulting in a replication error-prone (RER+) phenotype. Affected cells accumulate errors (mutations) at a much greater rate than normal cells (67). This is manifested clinically by a much faster progression of the disease. In contrast to sporadic colorectal cancers, which may take 10 to 15 years to develop, patients with HNPCC have been found to develop cancers within 2 years after a normal colonoscopy (68).

Microsatellite instability is not only a characteristic feature of HNPCC tumors, it is also observed in a subset (about 17%) of sporadic colorectal cancers (69–73). Sporadic tumors with microsatellite instability share common clinical and histopathological features with HNPCC tumors. They are usually located in the proximal colon (71–74), are associated with extracellular mucin production (73), poor differentiation (71–73), and diploidy (69, 72). In addition, they are less likely to have LOH at known tumor suppressor gene loci on chromosomes 5q, 18q, and 17p (74). On the other hand, inactivation of TGF-βRII due to frameshift mutations within coding microsatellite sequences occurs frequently (47, 48). Although defects in the same *MMR* genes that are affected in HNPCC have been identified in sporadic colorectal cancers with microsatellite instability (75), substantial differences in the nature and incidence of these mutations have been reported, suggesting that the molecular mechanisms underlying instability in the sporadic cases differ from those in HNPCC (2, 76). However, mutations in *hMSH3* and *hMSH6*, two other *MutS* genes initially identified in sporadic colorectal tumors with microsatellite instability (77, 78), have recently been reported in HNPCC patients. This suggests that the molecular mechanisms underlying instability may be similar in tumors with microsatellite instability regardless of their sporadic or hereditary nature (79).

EPIGENETIC FACTORS THAT CONTRIBUTE TO COLORECTAL CANCER

Age and diet. Consistent with the multi-hit hypothesis for the development of cancer, the incidence of colorectal cancer increases with age, as greater numbers of mutations are accumulated with time (80, 81). Although genetic aberrations and hereditary disorders play a critical role in the development of colorectal cancers (section above), the fact that the incidence varies tremendously according to geography suggests that other factors must also be involved (82). One of the major differences in lifestyle between cultures (which may explain geographic differences in colorectal cancer incidence) is diet (83). A number of dietary components have been implicated as possible factors in the development of colorectal cancer. The major factors include overall caloric intake, fat content of the diet, and fiber intake. The number of calories consumed per day and the fat content in the diet have been consistently positively correlated with the

risk of developing colorectal cancer (80). The mechanism by which a high-fat diet enhances tumor formation may be related to high fecal bile acid levels which stimulate mucosal epithelial proliferation. In contrast, vegetable and fiber consumption seem to have a protective effect (84, 85). Fiber may work either by increasing the stool transit time and thereby decreasing contact with fecal contents, by binding luminal toxic compounds, or by providing fuel for colonic bacteria that produce short-chain fatty acids that may inhibit proliferation and promote apoptosis (86). Consistent with the protective effects of dietary fiber, the intake of selenium, an essential trace mineral found in cereal grains, has also been found to be inversely correlated with the incidence of colorectal cancer (87–89). Since selenium is a cofactor for glutathione peroxidase, which participates in preventing free radical damage to tissues, part of selenium's protective effect may be due to a reduction in free radical damage.

Angiogenesis. In addition to the risk factors associated with lifestyle, physiological processes such as angiogenesis also contribute to the pathogenesis of colorectal cancer. Studies have shown that in order for a tumor to grow beyond a few millimeters in diameter, the formation of new blood vessels is required to provide nutrients and a means of eliminating metabolic waste products (90). As tumors get larger, the center of the tumor often becomes hypoxic due to inadequate vascularization, leading to cell death within the hypoxic center. Because hypoxia can induce apoptosis in a p53-dependent manner (91), low oxygen conditions can provide a selective advantage for cells carrying mutations in p53, allowing escape from apoptosis. This may be particularly important in colorectal cancer because p53 mutations are prevalent, and occur late in the adenoma-carcinoma sequence. Thus, by escaping apoptosis, tumor cells bearing p53 mutations within a colorectal carcinoma retain their proliferative capacity, thereby promoting tumor expansion. In addition, since p53 expression results in the secretion of inhibitors of angiogenesis (92, 93), selection of p53 mutations by hypoxic conditions could lead to loss of expression of antiangiogenic factors, allowing growth of new blood vessels, thereby favoring further expansion of the tumor.

Angiogenesis is not only essential for the expansion of the primary tumor, it is also required for the establishment and growth of metastases at distant sites. In fact, there is evidence that systemic suppression of angiogenesis can maintain micrometastases dormant as a result of a balance between proliferation and apoptosis (94). Therefore, the degree of angiogenesis may be an important factor in determining tumor behavior and the propensity to metastasize. Indeed, in patients with colorectal cancer, angiogenesis has been correlated with a higher recurrence rate and diminished survival (95).

Immune response. Another epigenetic factor important in the development of colorectal cancer is ineffective immune response. Unlike virally or chemically induced tumors, spontaneous tumors, such as those arising in the colon, elicit a weak immune response. Although mutant proteins encoded by oncogenes (K-*ras*) or tumor suppressor genes (*APC*, *DCC*, *Smad4*, *Smad2*, *tgf-βRII*, *tgf-β1*, *p53*) that have undergone mutations can be recognized as tumor-specific antigens, their recognition delivers only one of the two signals required for T-cell-mediated immunity. This is due to the fact that very few professional antigen-presenting cells (dendritic cells, macrophages, and B-cells) are present in colorectal tumors (96). Thus, most of the mutant proteins are processed and presented as antigenic peptides bound to class I MHC on the surface of colon cancer cells. Since these cells are not professional antigen-presenting cells, they lack the costimulatory signal (B7) that must be recognized by T-cells in order to elicit an immune response. Recognition of foreign antigen in the absence of a costimulatory signal leads to T-cell anergy. Consequently, although a large number of T-lymphocytes have been identified in primary colorectal tumors (97), T-cell-mediated immunity is ineffective in eliminating tumor cells. In contrast, natural killer cells have a spontaneous cytotoxic capacity against tumor cells. However, these cells are either not found or are only present in low numbers in colorectal cancers (96, 98). In addition, studies have shown that MHC expression on professional antigen-presenting cells within colorectal tumors is often lost, thereby further compromising immunity against tumors (99).

Related to immune function, epidemio-

logical studies have shown that chronic use of aspirin results in a reduced risk of colorectal cancer (100), and that treatment of FAP patients with non-steroidal anti-inflammatory drugs (NSAIDs) results in regression of rectal polyps (101). These studies suggest that inflammation may contribute to the development of colorectal cancer. NSAIDs mediate their effects by inhibiting two enzymes, COX-1 and COX-2, which are responsible for eicosanoid synthesis. Analysis of COX-2 mRNA in colon cancers and adenomatous polyps revealed increases of 86% and 43%, respectively, compared to in normal mucosa from the same patients (102). Consistent with elevated COX-2 levels, prostaglandin E2 levels have also been found to be elevated in colon cancers and polyps (103, 104). Further support for the role of COX-2 in the development of colorectal cancer has been obtained using an *APC* knockout mouse with a phenotype similar to that of the *Min* mouse. Inactivation of the *COX-2* gene in these mice resulted in an 86% decrease in intestinal and colonic polyps compared to control animals (105). In addition, when animals heterozygous for *APC*, but having normal *COX-2*, were treated with a novel specific inhibitor of COX-2 enzyme activity, polyp numbers decreased by 50–60% compared to a 26% decrease when they were treated with the NSAID sulindac (105). Moreover, feeding a selective COX-2 inhibitor to rats has been reported to completely prevent the development of chemically induced colon tumors in 93% of animals (106). These data suggest a direct role of COX-2 in the development of colorectal cancer.

There is evidence that the mechanism by which COX-2 overexpression contributes to colorectal cancer involves a decrease in apoptosis due to induction of Bcl-2 by prostaglandin E2, rather than an increase in inflammation, as initially suggested (107, 108). In addition, COX-2-overexpressing colon cancer cells have been shown to produce high levels of angiogenic factors, which stimulate both endothelial migration and tube formation (109). Thus, COX-2 may also act as a tumor promoter via stimulation of angiogenesis. This is consistent with a previous study which has shown that the antitumor effect of the COX inhibitor diclofenac was due to an antiangiogenic effect (110).

CONCLUSION

Since many genetic aberrations contribute to the development of colorectal cancer, each affected gene could potentially be a good therapeutic target. Identification of these genetic aberrations has provided an opportunity to use different strategies to deliver normal copies of defective tumor suppressor genes to affected tissues, or to inactivate oncogenes. Although these avenues hold promise, not all of the genetic aberrations described above occur in all individuals with colorectal cancer. In contrast, epigenetic factors do indeed contribute to the development of all colorectal tumors and thereby provide additional opportunities for intervention. Therapies targeting both genetic and epigenetic aberrations could be combined to provide a broader and more effective therapy for patients.

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Review

Transforming growth factor-β and breast cancer
Tumor promoting effects of transforming growth factor-β

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Abstract

The transforming growth factor (TGF)-βs are potent growth inhibitors of normal epithelial cells. In established tumor cell systems, however, the preponderant experimental evidence suggests that TGF-βs can foster tumor–host interactions that indirectly support the viability and/or progression of cancer cells. The timing of this 'TGF-β switch' during the progressive transformation of epithelial cells is not clear. More recent evidence also suggests that autocrine TGF-β signaling is operative in some tumor cells, and can also contribute to tumor invasiveness and metastases independent of an effect on nontumor cells. The dissociation of antiproliferative and matrix associated effects of autocrine TGF-β signaling at a transcriptional level provides for a mechanism(s) by which cancer cells can selectively use this signaling pathway for tumor progression. Data in support of the cellular and molecular mechanisms by which TGF-β signaling can accelerate the natural history of tumors will be reviewed in this section.

Keywords: transforming growth factor (TGF)-β, TGF-β receptors, epithelial-to-mesenchymal transition, angiogenesis

Introduction

Although the transforming growth factor (TGF)-βs can be tumor suppressive [1], there is increasing evidence that TGF-β secretion by tumor cells and/or stromal cells within the peritumoral microenvironment can contribute to tumor maintenance and progression. How, then, can TGF-βs be both tumor suppressive and tumor promoting? This apparent paradox is reconciled by a study showing that, in a mouse skin model of chemical carcinogenesis, targeted expression of TGF-β1 in suprabasal keratinocytes appears to have dual effects. It suppresses the formation of benign skin tumors, but once tumors develop, it enhances their progression to a highly invasive spindle cell phenotype [2••]. These results suggest that the effects of TGF-β1 are biphasic: TGF-β1 acts early as a tumor suppressor, probably by inhibiting the proliferation of nontransformed cells, and it acts later as a tumor promoter by eliciting an epithelial-to-mesenchymal transition (EMT). Additional experiments have suggested that upregulation of TGF-β3 in the spindle carcinomas was responsible for maintenance of this invasive phenotype [2••]. This is consistent with TGF-β3 expression at sites in mouse embryos where epithelial–mesenchymal interactions are important, like the lung and palatal shelves [3,4], and also the abnormal lung development and cleft palate observed in TGF-β3 null mice [5]. Also consistent with an early tumor suppressive effect is the recent observation that *tgf-β1−/−* mice develop an accelerated progression of epithelial hyperplasia to

CTL = cytotoxic T lymphocyte; EMT = epithelial-to-mesenchymal transition; JNK = c-Jun *N*-terminal kinase; MMP = matrix metalloproteases; PAI-1 = plasminogen activator inhibitor; PTHrP = parathyroid hormone-related protein; TβRI = TGF-β receptor type I; TβRII = TGF-β receptor type II; TGF-β = transforming growth factor-β.

colonic adenomas and cancers [6•]. The existence of dual effects for TGF-βs in tumor progression follows the observation that TGF-β-induced growth inhibitory responses and extracellular matrix responses may represent distinct processes in certain cell types. For example, overexpression of the antagonistic Smad, *Smad7*, in pancreatic carcinoma cell lines not only suppresses TGF-β1-mediated growth inhibition, but enhances the ability of TGF-β1 to induce matrix associated transcriptional responses [7•].

The progression of epithelial tumors to an invasive metastatic state is often associated with EMT, downregulation of cellular adhesion molecules, elevated expression of metalloproteases, and increased motility and angiogenesis, all of which can be modulated by TGF-βs. It is therefore not surprising that the TGF-βs can also promote tumorigenesis by modulating these critical processes. In support of this view, elevated levels of TGF-β are often observed in advanced carcinomas, and have been correlated with disease progression in several studies [8–13]. This suggests that secreting higher levels of TGF-β may provide an advantage to tumor cells. Both autocrine and paracrine signaling may be involved in conferring this selective advantage. While mutations in various components of the TGF-β signaling pathway have been observed in some carcinomas, particularly colorectal cancers [14,15], an intact TGF-β signaling pathway is often retained in other malignancies as some tumors can exhibit increased invasiveness in response to exogenous TGF-β [16,17•,18,19,20•,21]. Moreover, in a recent study of a large cohort of human breast tumors, loss or low levels of the type II TGF-β receptor (TβRII) correlated with high tumor grade, but 60% of *in situ* and invasive breast carcinomas retained robust levels of TβRII expression by immunohistochemistry [22]. Finally, although *Smad4* is frequently inactivated in pancreatic cancers [23,24], the *Smad* genes, which encode proteins that transduce TGF-β signals, are rarely mutated in most human carcinomas [25–30]. This suggests that after cells lose their sensitivity to TGF-β growth inhibition, autocrine TGF-β signaling may potentially promote tumor progression. In addition, TGF-βs produced in excess by tumor cells may act in a paracrine fashion on the peritumoral stroma, tumor neovessels, or the immune system, indirectly fostering tumor progression.

Autocrine effects

Epithelial-to-mesenchymal transition

Similar to keratinocytes [2••], TGF-β1 can also induce a rapid and reversible EMT in melanoma cells [31], and in both nontumor [32] and Ha-Ras transformed [17•] mammary epithelial cells *in vitro*. In Ha-Ras mammary tumors, EMT appears to be initiated by TGF-β produced by peritumoral host cells and later maintained by autocrine TGF-β1 as the converted tumor cells themselves begin to secrete TGF-β1. The Ha-Ras tumor cells obtained after EMT *in vitro* or *in vivo* display loss of epithelial polarity, downregulation of E-cadherin, disruption of cell–cell adhesion, and invasive properties in several *in vitro* assays [17•]. Supporting the importance of autocrine TGF-β for the tumorigenesis of Ha-Ras mammary cells, introduction of dominant negative TβRII into these cells retarded tumor formation and prevented EMT *in vivo*; moreover, introduction of the same construct into highly invasive murine colon carcinoma cells reconstituted an epithelial phenotype *in vitro*, and inhibited both tumor outgrowth and the establishment of metastases [20•]. In colon cancer cells of low invasive potential and with naturally occurring mutations in the TβRII gene, re-expression of TβRII function restored tumor cell invasiveness [20•]. In another study, expression of dominant negative TβRII in clones derived from a metastatic squamous carcinoma cell line prevented their spontaneous progression to a spindle phenotype *in vivo* [21]. Furthermore, approximately 90% of colon cancers with microsatellite instability have inactivating mutations of TβRII [33], and this instability is significantly correlated with longer patient survival [34], suggesting that complete loss of TβRII in carcinomas may limit systemic metastases. Taken together, these results suggest that EMT, local tumor growth, and metastatic progression can be sustained by autocrine TGF-β signaling.

When tumors are grown in nude mice, TGF-βs made by host cells can induce responses in tumor cells with intact TGF-β signaling, with the net effect of these tumor–host interactions being deleterious to the host. For example, MDA-231 human breast tumor cells secrete parathyroid hormone-related protein (PTHrP) in response to exogenous TGF-β1, metastasize to bone when injected into nude mice, and induce osteolysis and hypercalcemia, resulting in host death. Transfection of these cells with dominant negative TβRII blocks TGF-β1-mediated stimulation of PTHrP production. Mice injected with these cells exhibited less osteolysis, higher body weight, lower serum calcium and PTHrP levels, and longer survival than mice injected with control MDA-231 cells [35•]. On the contrary, accelerated osteolysis and reduced host survival were observed when mice where injected with tumor cells transfected with a constitutively active TβRI, suggesting a possible role for TGF-β-mediated responses in the pathogenesis of some adverse paraneoplastic syndromes.

Several recent studies have contributed to our understanding of the biochemical mechanisms by which transformed cells can lose autocrine growth inhibition but retain TGF-β-mediated responses that contribute to tumor progression. For example, oncogenic activation of the Ras pathway, acting via MAP kinases, causes phosphorylation of Smad2 and Smad3 at specific Erk consensus sites in the linker region between their DNA binding and transcriptional activation domains. This results in loss of nuclear accumulation of Smad2/3 and silencing of TGF-β-mediated

antiproliferative responses [36••]. In nontransformed mammary cells, introduction of mutant Ras not only blocks growth inhibition by TGF-β, but also subverts this pathway into one that can stimulate epithelial-to-mesenchymal transdifferentiation [17•,20•]. In MDCK epithelial cells, transfection of the missense mutations Smad2.D450E and Smad2.P445H, reported in primary colorectal and lung carcinomas, does not abolish TGF-β-mediated growth arrest. Instead, it increases both basal and TGF-β stimulated invasiveness, neither of which is prevented by overexpression of the inhibitory Smad7 [37•]. This suggests the existence of *Smad* 'gain-of-function' mutations that enhance malignant progression by mechanisms independent of TβRI and Smad phosphorylation. Another study has shown that Smad7 mRNA levels are increased in human pancreatic cancers compared with normal pancreas [7•]. Stable transfection of COLO-357 human pancreatic cancer cells with a Smad7 expression vector results in loss of TGF-β1-mediated growth inhibition and p21/Cip1 promoter activity. However, TGF-β1-induced plasminogen activator inhibitor-1 (PAI-1) promoter activity is maintained and, more importantly, basal PAI-1 promoter activity, PAI-1 mRNA levels, anchorage independent colony growth, and tumorigenicity in nude mice, are all increased in the Smad7 transfected clones [7•]. This result suggests another potential mechanism, the overexpression of Smad7, for the segregation between antiproliferative and matrix associated TGF-β responses. In addition, overexpression of Smad4 in colon carcinoma cells does not reconstitute TGF-β-mediated antiproliferative responses [38•,39], but inhibits cell adhesion and spreading, reduces the levels of urokinase plasminogen activator and PAI-1, and prolongs tumor latency [39], suggesting an additional function for Smad4 in restraining genes involved in peritumor proteolysis and invasion. This is further supported by reports of homozygous deletion of *TβRI* or homozygous missense mutations of *TβRII* [40,41], each coexisting with deletions of *Smad4* in individual tumors. The coexistence of these mutations in the same tumors would not be expected if the function of these two gene products (TβRII and Smad4 or TβRI and Smad4) was limited to a single common signal transduction pathway. Taken together, these studies suggest, first, that the threshold for loss of TGF-β antimitogenic effects is lower than that required to lose responses associated with cell adhesion, invasion, and metastases; second, that not one but multiple biochemical mechanisms can contribute to the enhancement or unmasking of the tumor promoting effects of autocrine TGF-β; and, third, that some of these mechanisms may be independent of Smad function or TβRI phosphorylation. The identification of Smad dependent and independent genes causally involved in these TGF-β-mediated tumor promoting effects requires further research. Of note, Hocevar *et al* [42•] recently reported c-Jun *N*-terminal kinase (JNK) dependent TGF-β-induced fibronectin expression in cell lines lacking the *Smad4* gene or protein expression.

Increased motility

TGF-β can stimulate the motility of many cell types *in vitro* [43–45], therefore suggesting that TGF-β production *in vivo* may enhance migration of tumor cells and metastatic potential. Indeed, cyclosporine treatment of lung adenocarcinoma cells results in increased cell motility and anchorage independent growth *in vitro*, as well as increased metastases *in vivo*, all of which can be blocked with neutralizing TGF-β1 antibodies [46]. These results suggest that *in vivo* tumor progression by cyclosporine is dependent on autocrine TGF-β1. In prostate cancer cells, TGF-β1 stimulates motility without affecting cell proliferation, suggesting that the effects on motility and proliferation may occur via different biochemical pathways [43].

Whether blockade of the Smad pathway, critical for TGF-β-mediated antimitogenic effects [47,48], is also critical for the effects of TGF-βs on cell motility is not clear. Some evidence suggests that the latter may follow alternative signaling pathways, perhaps in cooperation with activated oncogenes. Atfi *et al* [49] reported recently that inactivating components of the JNK pathway, which regulates AP-1 activity via c-Jun, inhibits TGF-β-mediated induction of 3TP-Lux, a reporter construct that contains Smad and AP-1 binding elements. Dominant negative mutants of RhoA, Rac1, and Cdc42, GTPases that mediate cell shape, cytoskeletal organization, and motility, abolish TGF-β-mediated transcription of AP-1 [49,50], suggesting that the Rho family of GTPases and the JNK pathway are essential components of TGF-β signaling responses. TGF-β1 can also upregulate integrin linked kinase [31], a protein associated with fibronectin production and increased cell motility. In another study, TGF-β1 treatment of NMuMG mouse mammary epithelial cells increased the expression of *N*-cadherin [51], which has been shown to increase motility of squamous cancer cells [52].

Paracrine effects

Induction of metalloproteases

Matrix metalloproteases (MMPs) play a critical role in the proteolytic degradation of basement membrane that is required for tumor invasion [53]. The expression of several MMPs, including MMP-2 [54] and MMP-9 [18,31,55], can be induced by TGF-β. Moreover, TGF-β1 has been shown to selectively induce MMP-9 activity in a subset of metastatic but not primary mouse prostate tumors, implying that this TGF-β1-induced response may be an important selection step in tumor progression [18]. There is also evidence that TGF-β increases MT-MMP-1 and MMP-9 expression in metastatic melanoma [31]. Although MMPs are listed separately, recent data implicate them strongly in the process of tumor-induced neovascularization [56], thereby suggesting that their upregulation might be an integral component of the TGF-β-mediated angiogenic processes discussed next.

Tumor angiogenesis

It is generally accepted that solid tumors require an adequate blood supply in order to grow beyond a few millimeters in size. TGF-βs, particularly TGF-β1, have been shown to regulate new blood vessel formation both *in vitro* and *in vivo* by a combination of responses that include increased production and facilitation of vascular endothelial growth factor, facilitation of basic fibroblast growth factor mediated capillary sprouting, inhibition of endothelial cell migration, and increased production of extracellular matrix, among others (reviewed in [57]). In most cells, TβRI/ALK-5 is the signaling receptor for TGF-β. However, in endothelial cells, it has been suggested that ALK-1 may also function as a type I receptor for TGF-β [58]. In addition to the type I, II, and III TGF-β receptors, endoglin is another integral membrane protein that binds TGF-β1 and TGF-β3, and is highly expressed in endothelial cells [59]. Although TGF-β effects appear to be mediated mostly by the receptor specific Smad2 and Smad3 proteins [47,48], there is evidence that Smad5 is involved in TGF-β signaling in hematopoietic cells [60]. Targeted disruption of genes encoding various components of the TGF-β signaling pathway, including TGF-β1 itself [61], its receptors, TβRII [62], ALK-1 [63], and endoglin [64], and one of its signal transducers, Smad5 [65], has each revealed that these proteins play an important role in vascular development. The phenotype of the TGF-β1 and TβRII knockout mice is virtually indistinguishable and is characterized by defective endothelial differentiation resulting in abnormal capillary tube formation [61,62]. In contrast, disruption of ALK-1, endoglin, or Smad5 does not affect endothelial differentiation or vasculogenesis, but instead they each affect angiogenesis. In addition, *endoglin−/−* and *Smad5−/−* mice exhibit impaired vascular smooth muscle cell development. These results are consistent with previous reports demonstrating that TGF-β can regulate smooth muscle cell differentiation and migration *in vitro* [66•], thus contributing to pericyte recruitment and vessel stabilization. This hypothesis, as it applies to tumor angiogenesis, is somewhat challenged by the notion that the majority of intratumoral neovessels seem to lack periendothelial smooth muscle cells [67], suggesting that there may be additional roles for the TGF-βs in tumor angiogenesis. In that light, Higaki and Shimokado [68] recently reported TGF-β1-mediated stimulation of phosphatidylinositol-3 kinase activity and amino acid uptake in vascular smooth muscle cells, suggesting a direct anti-apoptotic role for TGF-β. Elucidation of the paracrine mechanisms driving TGF-β-mediated tumor angiogenesis requires further investigation.

Further supporting the role of TGF-βs in tumor angiogenesis, administration of a neutralizing TGF-β1 antibody to nude mice harboring CHO cell xenografts transfected with ectopic TGF-β1 inhibits both tumor growth and intratumor microvessel density [69]. In addition, a monoclonal antibody that blocks TGF-β1, TGF-β2, and TGF-β3 has been shown to suppress the growth of TGF-β1-overexpressing renal cancer xenografts [70]. In this study, the TGF-β blocking monoclonal-abrogated factor VIII staining in the xenografts, suggesting an antitumor mechanism that targets endothelial cells [70]. Furthermore, TGF-β1 and PAI-1 have been shown to inhibit the conversion of plasminogen to the anti-angiogenic molecule angiostatin in medium conditioned by human pancreatic cancer cells [71]. This suggests an additional pro-angiogenic mechanism for TGF-β by interfering with the production of endogenous inhibitors of endothelial cell proliferation. Finally, high levels of TGF-β1 mRNA correlate strongly with high microvessel density in breast tumors, and each of these factors is associated with poor patient outcome [72].

Host immunosuppression

TGF-β1 and TGF-β2 are potent immunosuppressants [73]. Thus, elevated levels of TGF-βs secreted by tumors could potentially inhibit immune effector cells and favor tumor progression. In support of this idea, Torre Amione *et al* [74] demonstrated that, unlike parental tumor cells, fibrosarcoma cells transfected to express 10 ng/ml TGF-β1 *in vitro* are unable to induce cytotoxic T lymphocyte (CTL) responses and can escape immune recognition. Likewise, EMT6 mammary tumor cells, which produce high levels of TGF-β1, can inhibit CTLs *in vivo*. Transfection of these cells with interleukin-2, a known T cell growth factor, can reverse this TGF-β1 effect and induce tumor rejection [75]. This result suggests that, by dampening the generation of tumor reactive T cells, TGF-β can promote tumor viability. There is also evidence that overexpression of the soluble TβRII extracellular domain in thymoma cells can prevent the progression of unmodified thymoma cells when injected near the primary tumor inoculation site [76], further suggesting that secretion of soluble TβRII by these cells is sufficient to restore tumor specific cellular immunity and mediate partial tumor rejection. Overall, these results are consistent with the phenotype of *TGF-β1* null mice that die shortly after birth as a result of widespread inflammation and multiorgan T cell infiltration and necrosis [77].

In addition to inhibiting CTL responses, TGF-βs can modulate other immune functions that may favor tumor progression. For example, CHO cells transfected with an expression vector encoding latent TGF-β1, when injected into nude mice, can decrease mouse spleen natural killer activity and rapidly form tumors [78]. Antagonizing TGF-βs by intraperitoneal injection of an antibody that neutralizes TGF-β1, TGF-β2, and TGF-β3 has the opposite effect. It prevents tumor and metastases formation by MDA-231 human breast carcinoma cells, and markedly increases natural killer activity of mouse splenocytes [79]. Consistent with this TGF-β-mediated immunosuppressive effect, reduced immune function has been observed in animals

bearing TGF-β overexpressing tumors [80] as well as in patients with glioblastoma, a common type of brain tumor that frequently overexpresses TGF-β2 [81].

The cited studies suggest that tumor cell secreted TGF-βs may block the efferent function of immune effectors at sites of tumor implantation. Other reports, however, suggest tumor cell TGF-βs may modify the afferent component of the immune response and confer antitumor immunity. Stable infection of breast and glioma tumor cells with antisense TGF-β1 and antisense TGF-β2 retroviruses, respectively, has been shown to restore the immunogenicity of these tumor cells when injected into immunocompetent animals. Furthermore, they induce a partial rejection of unmodified, less immunogenic established wild type tumor cells [82,83]. In both of these studies, *in vitro* and *in vivo* CTL activity was markedly increased in medium conditioned by antisense TGF-β-infected cells and/or in mice injected with tumor cells bearing the antisense compared with tumor cells infected with a control vector. These studies have therapeutic implications for the use of an antisense TGF-β based approach as a means of adoptive immunotherapy against TGF-β overproducing tumors.

Alternative views and conclusions

A tumor permissive role for the TGF-βs may not apply to all solid tumors. Indeed, transfection of an antisense TGF-β1 expression vector into FET and CBS well-differentiated human colon cancer cells has been shown to enhance tumor formation in nude mice [84,85], supporting the notion that, in some fully transformed cells, endogenous TGF-β1 can continue to mediate a tumor suppressor function. In a recent report, mice bearing transplanted gallbladder Mz-Cha-2 tumors showed inhibition of angiogenesis and leukocyte–endothelial cell interactions at a distant cranial site and threefold higher levels of circulating TGF-β1 compared with tumor free mice [86]. This reduction in microvessel density and leukocyte rolling were reversed by systemic administration of a TGF-β1 neutralizing antibody, suggesting a negative role for TGF-β1 in early neovascularization. Moreover, in a recent survey of 104 *in situ* and invasive primary breast carcinomas, 40/45 (89%) tumors with low invasive potential and low proliferation rate exhibited high levels of TβRII by immunohistochemistry [22]. Whether autocrine TGF-β signaling is causally associated with the observed low proliferation and invasiveness in this subset of breast tumors is a question that remains unclear.

Nonetheless, the potential tumor promoting effects of TGF-β provide novel molecular targets for interventions aimed at altering the natural history of solid tumors. The lack of an obvious physiological role for TGF-β signaling in postdevelopmental normal physiological states suggests that these interventions may in fact be tumor specific and spare the tumor host from undue toxicity. Several approaches have been proposed, including the use of blocking antibodies against TGF-β1, TGF-β2, and TGF-β3, using the soluble ectodomains of the type II and III TGF-β receptors, which would sequester TGF-β isoforms at tumor sites and prevent binding to cognate receptors [87,88], and, finally, using adenovirus encoding inhibitors of TGF-β signaling [89], to name a few. The theoretical and logistical strengths and limitations of these approaches are beyond the scope of this review. Nonetheless, these represent tools that, if effective in blocking TGF-β action, will allow us to address the net effect of autocrine/paracrine TGF-β signaling at early and late stages of transformation and cancer progression.

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INVASION AND METASTASIS OF A MAMMARY TUMOR INVOLVES TGF-β SIGNALING

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Several studies have correlated escape from TGF-β-mediated cell cycle arrest with the tumorigenic phenotype. Most often, this escape from growth control has been linked to dysfunctional TGF-β receptors or defects in the TGF-β-mediated SMAD signaling pathway. In this report, we found that highly metastatic 4T1 mammary carcinoma cells express functional TGF-β receptors capable of initiating SMAD-mediated transcription, yet are not growth inhibited by TGF-β1. We further observed that TGF-β directly contributes to the metastatic behavior of this cell line. Exposure to TGF-β caused 4T1 cells to undergo morphological changes associated with the metastatic phenotype and invade more readily through collagen coated matrices. Furthermore, expression of a dominant negative truncated type II receptor diminished TGF-β signaling and significantly restricted the ability of 4T1 cells to establish distant metastases. Our results suggest that regardless of 4T1 resistance to TGF-β-mediated growth inhibition, TGF-β signaling is required for tumor invasion and metastases formation.

Key words: *TGF-β; Smad; metastasis; mammary cancer; 4T1*

Tumor invasion and metastasis are complex processes in which cancer cells detach from the original tumor mass to establish metastatic foci at distant sites. Metastatic cells characteristically lose growth inhibitory responses, undergo alterations in adhesiveness and demonstrate enhanced production of enzymes that can degrade extracellular matrix components.[1,2] Since it is the development of metastatic disease that is primarily responsible for cancer mortality, an understanding of the mechanisms that facilitate metastatic tumor progression is of great importance.

One cytokine that may contribute to the metastatic potential of tumor cells is transforming growth factor beta (TGF-β). Originally identified as a positive growth factor for mesenchymal cells, TGF-β has been identified as a potent growth inhibitor of most cell types, including cells of hematopoetic origin and epithelial lineage.[3] TGF-β inhibits the growth of normal epithelial cells by inducing an arrest in G1 phase of the cell cycle and, less commonly, by promoting apoptosis.[3,4] In contrast, most malignant cells are refractory to TGF-β-mediated growth arrest[5]. This loss of sensitivity has been linked to tumor progression, and may be due to loss or mutation of TGF-β receptors or dysregulation of TGF-β signal transduction pathways.[2,4,6]

It is widely accepted that TGF-β promotes tumorigenicity by stimulating angiogenesis,[7] inducing extracellular matrix degradation[8] and inhibiting anti-tumor immune responses.[9–12] Recent studies have shown that tumorigenicity can also arise via the action of TGF-β on the tumor cells directly.[12–19] In this report, we have employed a highly metastatic murine mammary cancer cell line (4T1) to examine the role of TGF-β on invasion and metastatic potential *in vivo*. We found that although 4T1 mammary tumor cells are resistant to TGF-β-mediated growth inhibition, TGF-β signaling is critical to tumor invasion and metastases formation. Engagement of ligand initiated downstream signaling pathways that culminated in Smad2 phosphorylation and transcription of a Smad responsive reporter gene (3TP-lux). Furthermore, disruption of TGF-β signaling by expression of a dominant negative truncated type II receptor significantly curbed tumor metastasis without affecting primary tumor growth.

MATERIAL AND METHODS

Cell lines

The metastatic line 4T1 is a thioguanine-resistant variant of 410.4, a tumor subline isolated from a spontaneous mammary tumor that developed in a BALB/cfC3H mouse. The 4T1 cell line was kindly provided by Dr. Fred Miller of the Michigan Cancer Foundation (Detroit, MI). The TGF-β sensitive Mink lung epithelial cell line (Mv1Lu) was purchased from ATCC (CCL64; Rockville, MD). Both cell lines were maintained *in vitro* by passage in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS).

Mice

Female C.B-17/IcrACC*scid* mice, 6–8 weeks old, were purchased from a colony maintained at the University of Arizona. The mice were housed in the University of Arizona animal facilities in accordance with the principles of animal care (NIH publication No. 85-23, revised 1985).

Cell cycle analysis

4T1 tumor cells and TGF-β sensitive Mv1Lu cells were arrested at the G1 phase of the cell cycle by seeding them at high density (10^7 cells/T75 flask) for 72 hr. The cells were then detached and plated at a concentration of 5×10^5 cells/100 mm × 15 mm tissue culture dish in the presence of increasing amounts of TGF-β1. Forty-eight hours later, the cells were collected, fixed at a concentration of 10^6 cells/ml with cold 70% ethanol for 2 hr and stored at 4°C for up to 1 week. For analysis, 10^6 cells were suspended in 1 ml of Vindelov's PI buffer pH 8.0 (10 mM Trizma base, 10 mM NaCl, 0.1% NP-40, 50 μg/ml RNase A and 50 μg/ml propidium iodide). After a 20 min incubation at room temperature, the cells

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were filtered through a 30 μm nylon mesh and measured for DNA content with a FACStar^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems; San Jose, CA) using an Innova 90-5 argon laser (Coherent; Palo Alto, CA) tuned to 488 nm at 100 mW. Fluorescence emission was captured through a 575/26 nm bandpass filter. Data were acquired using Lysys II software and analyzed with CELLFIT (Becton Dickinson Immunocytometry Systems).

Determination of TGF-β receptor expression

Reverse-transcriptase polymerase chain reaction (rt-pcr). Total RNA was isolated from 4T1 and Mv1Lu cells with TRIzol reagent (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD), treated with DNase I and reverse transcribed to generate cDNA (cDNA cycle kit number K1310-02, Invitrogen; San Diego, CA). PCR amplification was performed in a 50 μl volume containing 2 μl of the cDNA reaction, 1.5 mM $MgCl_2$, 200μM dNTPs, 0.1μM sense and antisense primers for TGF-βRI or TGF-βRII, and 2.5 units Amplitaq Gold DNA polymerase (N808-0241, PE Applied Biosystems; Foster City, CA) in 1× PCR buffer. Cycling parameters were 95°C for 10 min followed by 94°C for 10 sec, 65°C for 30 sec and 75°C for 45 sec for 50 cycles. TGF-βRI- and TGF-βR-II specific primers were designed using the OLIGO program (National Biosciences; Plymouth, MN) and were as follows: TGF-βRI upper primer (5′-GGGGCGAAGGCATTACAGTG-3′, position 76) and TGF-βRI lower primer (5′-ATTTGGCACACGGTGGTGAA-3′, position 461); TGF-βRII upper primer (5′-TCCACGTGCGCCAACAACAT-3′, position 915) and TGF-βRII lower primer (5′-GCGCAAGGACAGCCCGAAGT-3′, position 1420). These primers amplify 405 bp and 525 bp fragments of TGF-βRI and TGF-βRII, respectively.

^{125}I-TGF-β1 affinity crosslinking studies. Subconfluent 4T1 tumor cells in 12-well plates were labeled with 100 pM ^{125}I-TGF-β1 (173μCi/μg, DuPont NEN; Boston, MA) in the presence or absence of 5nM unlabeled rhTGF-β1 (Genentech, South San Francisco, CA) for 4 hr at 4°C. After washing, the specifically bound labeled ligand was cross-linked to cell surface receptors with 1 mM bis(sulfosuccinimidyl) suberate (BS^3) (Pierce; Rockford, IL) as described previously.[20] Equivalent amounts of protein were then resolved by 3–12% gradient SDS-PAGE and labeled receptors visualized by autoradiography.

Cell migration and invasion assays

Cells were seeded at a density of 10^5 cells per well in triplicate in the upper chamber of 12-well transwells (8μm pore, number 29442-120, Corning, Inc., Corning, NY) in the presence or absence of 2 ng/ml rhTGF-β1 or anti-TGF-β neutralizing antibody (100 μg/ml) (ATCC HB-9849). Twenty hours later, the transwells were removed and the lower chambers were incubated for an additional 20 hr. Cells that migrated through the transwell into the lower chamber and attached were treated with trypsin and counted. The transwells were untreated (migration assay) or coated with rat tail type I collagen (invasion assay) as previously described.[21]

Confocal microscopy

Cells (10^4) were grown on 0.17 mm thick coverslips in a CO_2-humidified incubator for 48 hr in the presence or absence of 2 ng/ml rhTGF-β1. The cells were then fixed with 4% methanol-free formaldehyde for 20 min at room temperature, permeabilized with 100% methanol at −20°C for 6 min, air-dried and stored at −20°C until time of staining. Morphological changes in tumor cells treated with TGF-β were detected by staining the cells with 100 μl of a 1:40 dilution of bodipy-phalloidin (number B-3416, Molecular Probes, Eugene, OR), a fluorescent molecule that binds to actin filaments. The cells were visualized with a LEICA confocal microscope.

Western blotting

Cells grown overnight in serum free IMDM were untreated or treated with 0.25 ng/ml of rhTGF-β1 for 45 min, washed twice with ice-cold PBS and scraped into a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin. The lysate was placed on a rocker at 4°C for 30 min, centrifuged at 14,000g and protein in the supernatant recovered and quantified by BCA Protein Assay (Pierce, Rockford, IL). Proteins (25 μg) from the cell lysates were resolved by SDS-8% PAGE and electrotransferred to polyvinylidene fluoride (PVDF) membrane. Nonspecific binding sites were saturated by incubation in TBS containing 0.1% Tween-20 and 5% nonfat powdered milk. Membrane bound anti-Smad2 and anti-phosporylated Smad2 antibodies (S66220; Transduction Laboratories, Lexington, KY and 06-829; Upstate Biotech, Lake Placid NY, respectively) were visualized with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech) and enhanced chemiluminescence (Amersham Pharmacia Biotech, UK).

Plasmids

A plasmid construct containing cDNA encoding a truncated TGF-βRII lacking the serine/threonine kinase domain (DNRII)[22] was kindly provided by Dr. Barbara Ballerman of The Johns Hopkins University. For our studies, the 0.58 kb DNRII fragment was excised by a Nhe1/Xho digest and cloned into a commercially available plasmid pcDNA3.1zeo(+) (V860-20, Invitrogen). Proper insertion of this fragment into this plasmid was confirmed by restriction analysis and sequencing. The resultant construct (DNRII-pcDNAzeo) contained the truncated receptor under control of human CMV promoter and a SV40-driven zeocin resistance gene to allow for selection of stably transfected cells. The p3TPLux TGF-β-inducible luciferase construct was a gift from Dr. Joan Massagué (Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY) and has been described previously.[23] The β-galactosidase expression vector VR1412 used to normalize luciferase activities was kindly provided by Vical Inc. (San Diego, CA).

Transcriptional response assay

Cells were plated to semiconfluency and 24 hr later were transiently co-transfected with 1 μg each of p3TPLux and a control vector (VR1412) with Lipofectamine reagent (number 18324-012, GIBCO BRL) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were plated in triplicate at a concentration of 5×10^4 cells/well of a white 96-well culture plate (number 6005180, Instrument Company, Inc., Meriden, CT) in the presence or absence of 2 ng/ml rhTGF-β1 for 18 hr. Extracts were then prepared and assayed for luciferase and β-galactosidase activity using the LucLite Plus (number 6016961, Packard) and Galacto-Light Plus (BL100P, Tropix, Bedford, MA) kits, respectively. Light emission was detected with a Packard Lumicount and luciferase activity was normalized on the basis of β-galactosidase expression.

Northern blot analysis

Messenger RNA was isolated from subconfluent cells and subjected to Northern blot analysis using a NorthernMax-Gly kit (number 1944; Ambion, Inc., Austin, TX). Briefly, the mRNA was separated on a 1% agarose gel, transferred to nylon membrane, UV crosslinked and hybridized with biotin-labeled rat TGF-β receptor II and mouse GAPDH-specific RNA probes overnight at 80°C and 65°C, respectively. Hybridized bands were visualized by the addition of streptavidin/alkaline phosphatase using a BrightStar Biodetect kit (number 1925; Ambion, Inc.). To develop an antisense TGF-β receptor II (TβRII) template for transcription of a TβRII-specific probe, the DNRII fragment obtained from a Nhe1/Xho1 digest was cloned into pcDNA3.1(+) that had been digested with Xho1 and XbaI. To generate the sense TβRII RNA probe, this template was linearized by Apa1 digestion, treated with proteinase K and purified on a 4% polyacrylamide gel. The linearized plasmid was transcribed with T7 polymerase in the presence of a nucleotide triphosphate RNA-labeling mix (number 1685 597; Roche Molecular Biochemicals, Indianapolis, IN) containing biotin-16-UTP

according to the manufacturer's instructions. Biotin-labeled RNA probe specific for GAPDH and RNA millennium markers were similarly generated from linearized templates (number 7431 and number 7785, respectively; Ambion, Inc.).

Spontaneous metastasis assay

Mice challenged orthotopically in the mammary gland with 10^5 cells were sacrificed 21 days post-injection. Lungs were removed, stained with India ink and the tumors bleached with Fekete's solution as previously described.[24] Primary tumors were measured in 2 dimensions every 3–4 days post inoculation. Tumor volume was calculated using the formula $v = (l \times w^2)/2$, where v = volume (mm^3), l = long diameter and w = short diameter[24].

RESULTS

TGF-β does not inhibit cell cycle progression of 4T1 cells

We assessed whether exposure of 4T1 tumor cells to TGF-β resulted in growth inhibition. For this purpose, cells were arrested at G1 by crowding, released to transit through the cell cycle in the presence or absence of increasing amounts of TGF-β and then analyzed by flow cytometry. As shown in Figure 1, 4T1 cells were not growth arrested in G1 but instead progressed through the cell cycle in the presence of high concentrations (20 ng/ml) of TGF-β1. In contrast, indicator Mink lung epithelial cells (Mv1Lu) that are growth inhibited by TGF-β were arrested in G1 following exposure to 2 ng/ml TGF-β1 (Fig. 1). The resistance of 4T1 cells to TGF-β growth control was additionally supported by cell counting; no differences in cell number and viability were observed between untreated ($2.3 \times 10^6 \pm 3.6 \times 10^5$) and TGF-β-treated cells ($2.4 \times 10^6 \pm 1.5 \times 10^4$) after 48 hr of *in vitro* culture.

Expression of functional TGF-β receptors in mammary tumor cells

Since 4T1 cells were resistant to TGF-β-mediated cell cycle control, we examined them for the presence of functional TGF-β receptors. Analysis of RNA by RT-PCR demonstrated that 4T1 cells transcribe mRNA for TGF-β receptors I and II (Fig. 2*a*). The PCR product obtained for TGF-βRI was sequenced and determined to be ALK-5 (data not shown). 4T1 cells also express TGF-βRI and TGF-βRII as surface proteins capable of binding TGF-β ligand. When labeled with ^{125}I-TGF-β1 in a receptor cross-linking experiment, TGF-β receptors I, II and III were detected in 4T1 cells (Fig. 2*b*). Binding of ^{125}I-TGF-β1 was competitively inhibited by the addition of an excess of unlabeled TGF-β1 (Fig. 2*b*).

TGF-β signaling in 4T1 tumor cells

Next we evaluated if TGF-β signaling occurred in 4T1 tumor cells following TGF-β treatment. The involvement of Smads in TGF-β signaling was determined by Western blot to detect the presence of phosphorylated Smad-2 and by gene expression analysis of cells transiently transfected with the Smad responsive 3TP-Lux reporter construct. Smad2 phosphorylation and gene transcription in unstimulated 4T1 cells were significantly increased upon addition of exogenous TGF-β1 (Fig. 3).

Morphological changes induced by TGF-β treatment

The 4T1 mammary tumor cell line typically adheres to plastic or glass substratum and grows in rounded clumps (Fig. 4*a*). When incubated with 2 ng/ml rhTGF-β1, the cells became less clumped, assumed a fibroblast-like appearance and demonstrated reorganization of the cytoskeleton characterized by prominent F-actin filaments and stress fibers (Fig. 4*b*). In order to determine if this morphologic change involved an epithelial to mesenchymal transition (EMT), cells were stained with vimentin and keratin antibodies as previously described.[25] 4T1 cells constitutively co-expressed vimentin and keratin intermediate filaments and TGF-β treatment did not alter their expression (data not shown). This interconverted phenotype has been previously reported and is characteristic of highly malignant breast cancer cells.[25,26–28]

TGF-β stimulates in vitro migration and invasion of 4T1 cells

Since SMAD-mediated-TGF-β signaling was intact in 4T1 cells and TGF-β treatment induces morphologic changes, we speculated that TGF-β might contribute to tumor progression by stimulating cell migration and invasiveness. To explore this possibility, the effect of TGF-β on *in vitro* migration and invasion of 4T1 cells was evaluated. The data (Fig. 5) demonstrated basal levels of migration but negligible invasive capacity in 4T1 cells in the absence of TGF-β. Addition of TGF-β resulted in a 4-fold increase in *in vitro* migration and a 17-fold increase in invasiveness that was completely abrogated in the presence of TGF-β neutralizing antibody (Fig. 5).

Disruption of TGF-β signaling inhibits metastasis formation

To directly evaluate the role of TGF-β signaling in tumorigenicity and the development of metastasis, 4T1 cells were trans-

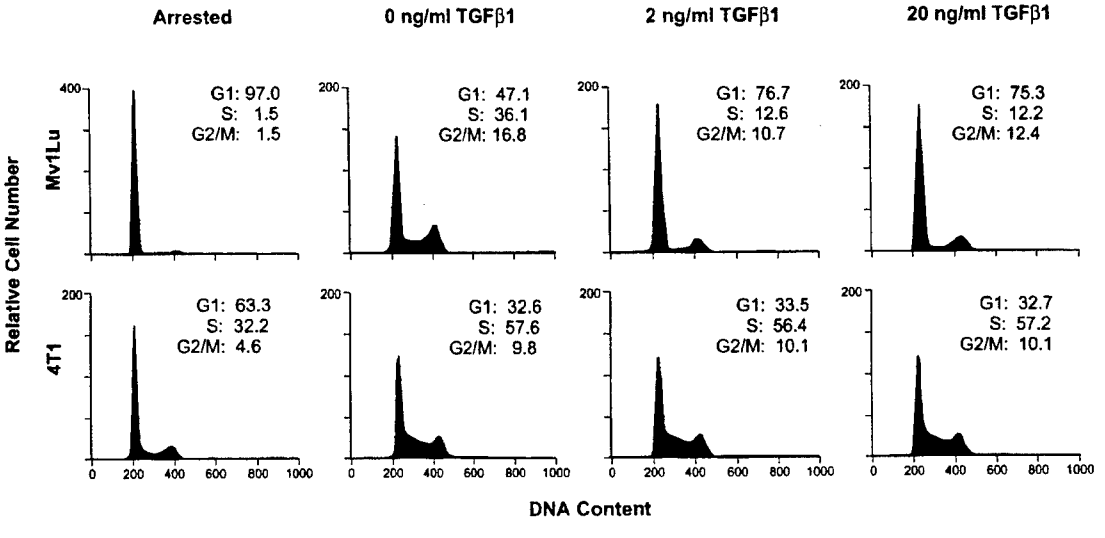

FIGURE 1 – Effect of TGF-β on the cell cycle of 4T1 cells. Mv1Lu and 4T1 cells arrested in G1 by crowding were released from growth arrest by detaching and plating at a lower cell density in the presence of different concentrations of rhTGF-β1. After 48 hr, the cells were collected and evaluated for DNA content by flow cytometric analysis as described in Material and Methods.

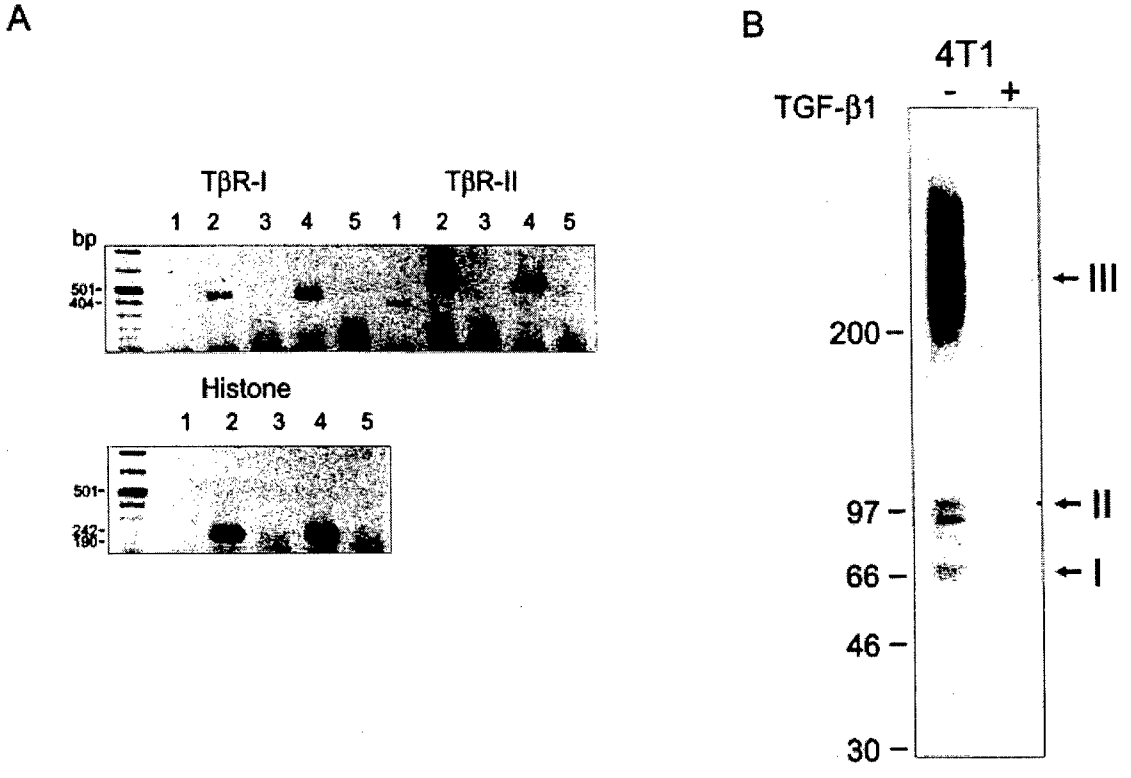

FIGURE 2 – Expression of TGF-β receptors by 4T1 tumor cells. (*a*) RT-PCR was performed on total RNA isolated from 4T1 (lane 2) and Mv1Lu cells (lane 4) using primers specific for a 405 bp fragment of TGF-βRI, a 525 bp fragment of TGF-βRII or a 215 bp fragment of histone. Lanes 1, 3 and 5 represent PCR reactions containing no template, 4T1 RNA or MvLu RNA (no RT) respectively. (*b*) Subconfluent 4T1 tumor cells were labeled with 100 pM ^{125}I-TGF-β1 in the presence (+) or absence (−) of 5 nM competing unlabeled TGF-β1. Bound labeled ligand was cross-linked to cell surface receptors, the proteins resolved by SDS-PAGE and labeled receptors visualized by autoradiography.

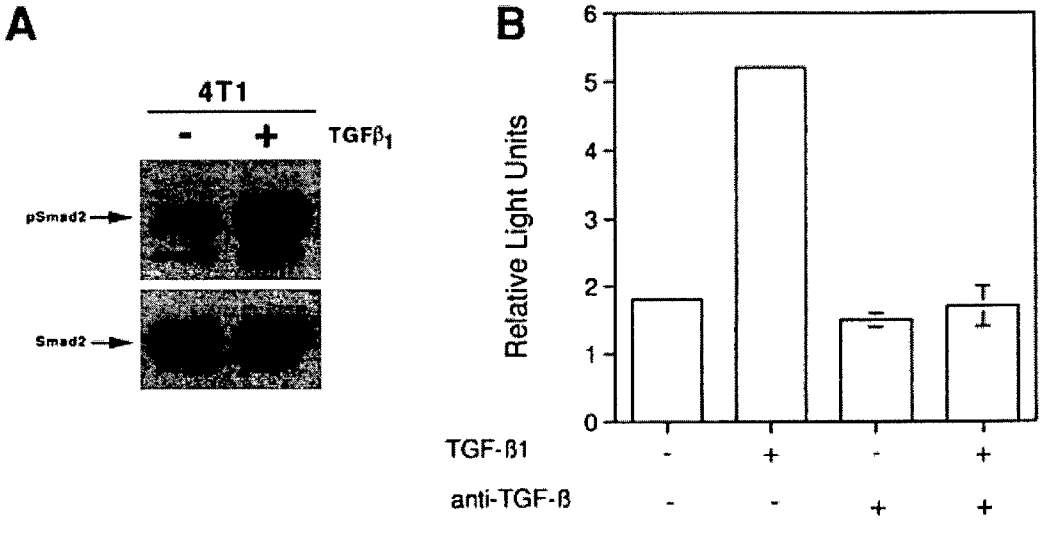

FIGURE 3 – TGF-β induction of Smad2 phosphorylation and transcriptional responses in 4T1 cells. A. Cells were incubated in the presence (+) or absence (−) of 2 ng/ml rhTGF-β for 45 min. Cell lysates were separated by SDS-PAGE, transferred to membrane and incubated with pretested dilutions of antibodies specific for Smad2 or the phosphorylated form of Smad2 (55 kDa). (*b*) 4T1 cells cotransfected with p3TP-lux reporter and VR1412 β-galactosidase control plasmids were treated with 2 ng/ml rhTGF-β1 and/or anti-TGF-β neutralizing antibody 18 hr before determination of luciferase activity. Bars represent the mean relative light units and standard deviation of triplicate samples. Luciferase activities were normalized based on β-galactosidase expression.

fected with a truncated dominant-negative TGF-β type II receptor (DNRII). This receptor lacks the cytoplasmic serine/threonine kinase domain and is therefore unable to transduce TGF-β-initiated signals[22]. Expression of DNRII was demonstrated by Northern blot (Fig. 6) and 4 of the stably transfected clones were selected for further analysis. Expression of DNRII blunted tran-

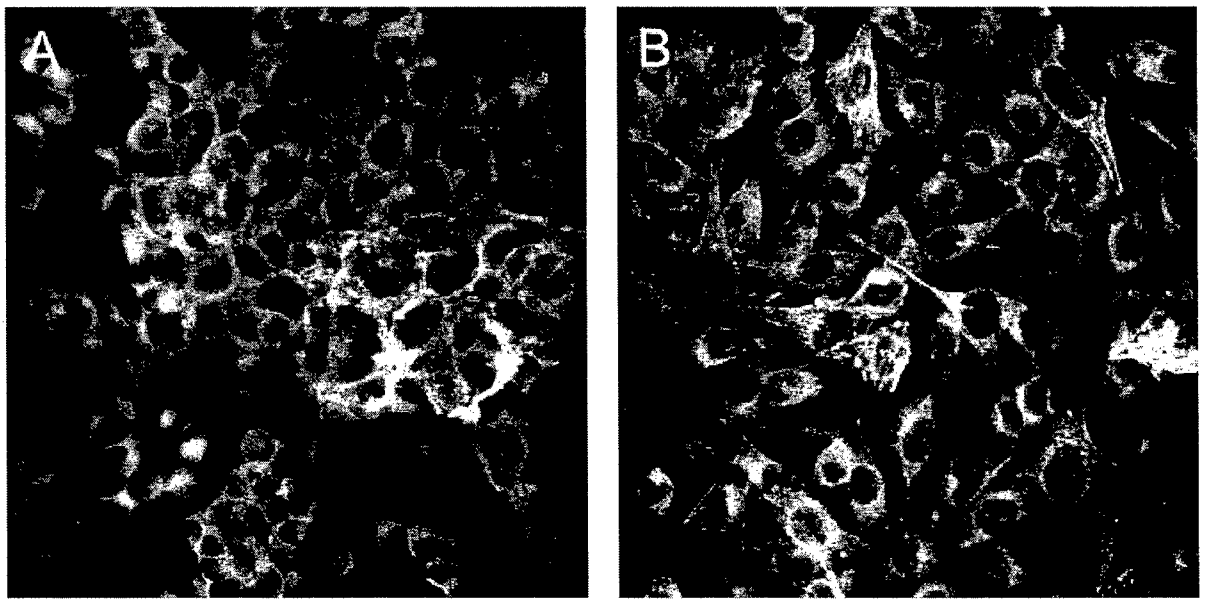

FIGURE 4 – Effect of TGF-β on tumor cell morphology. Subconfluent 4T1 cells were grown on glass coverslips for 48h in culture medium in the absence (*a*) or presence (*b*) of rhTGF-β1. Cells were visualized with a Lieca confocal microscope after staining actin fibers with bodipy-phalloidin. Magnification = 400×.

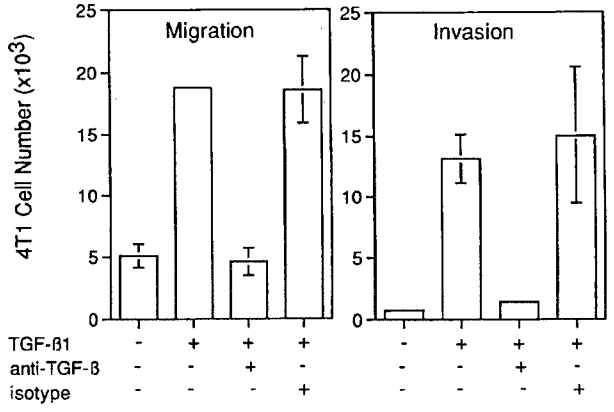

FIGURE 5 – TGF-β1 enhances migration and invasion of 4T1 cells. Tumor cells were placed in the upper chamber of 12-well transwells and incubated in the presence of anti-TGF-β antibody, rat IgG (isotype) or rhTGF-β1. Cells that moved through untreated transwells (migration assay) or collagen coated transwells (invasion assay) into the lower chamber were counted. Bars represent the mean cell number ± SE of duplicate wells. The data shown are representative of at least 2 experiments.

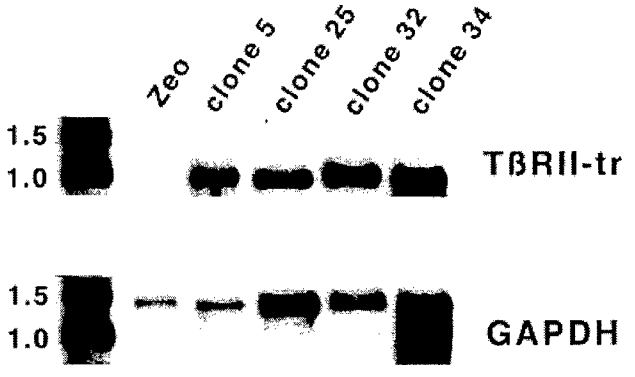

FIGURE 6 – Expression of dominant negative truncated TGF-β receptor II in transfected 4T1 cells. mRNAs isolated from mock-transfected control cells (Zeo) and 4T1 cells stably transfected with DNRII-pcDNAzeo (clones 5, 25, 32 and 34) were subjected to Northern blot hybridization with TGF-β receptor II and GAPDH-specific RNA probes.

scriptional activation by TGF-β in the majority of clones tested (Fig. 7). Additionally, the expression of DNRII resulted in a decrease of TGF-β-induced phosphorylation of Smad2 (Fig. 7*b*). Furthermore, when injected orthotopically into the mammary gland of immunodeficient SCID mice, 3 of the 4 clones expressing the DNRII were severely inhibited in their ability to form metastatic lung nodules (Table I). Approximately 114 ± 35 (n=4) metastatic nodules were found in the lungs of mice that were orthotopically injected with mock-transfected 4T1 cells (4T1Zeo) compared with 15.5 ± 6.5 (n=15) in mice injected with DNRII-expressing tumor cells. In the majority of mice injected with gene-modified tumor cells, the sizes of the primary tumors were not significantly different from those in control animals injected with tumor cells transfected with vector alone (Table I). Histologically, no discernible differences in the morphology or local invasiveness of the primary tumors were observed among the test and control groups. All primary tumors were well demarcated and extended from the epidermal/dermal junction through the dermis with infiltration into the abdominal musculature. Each tumor was bordered by mild neutrophilic inflammatory infiltrates and contained extensive necrotic foci. Carcinoma cells were not observed in blood vessels adjacent to the primary tumors. In contrast to the effects on *in vivo* metastasis, the expression of the truncated TGF-β type II receptor did not affect *in vitro* migration and invasion (data not shown).

DISCUSSION

In our study we investigated the role of TGF-β in the metastatic phenotype of a highly metastatic murine 4T1 mammary tumor. We found that TGF-β contributes to the ability of the tumor cells to

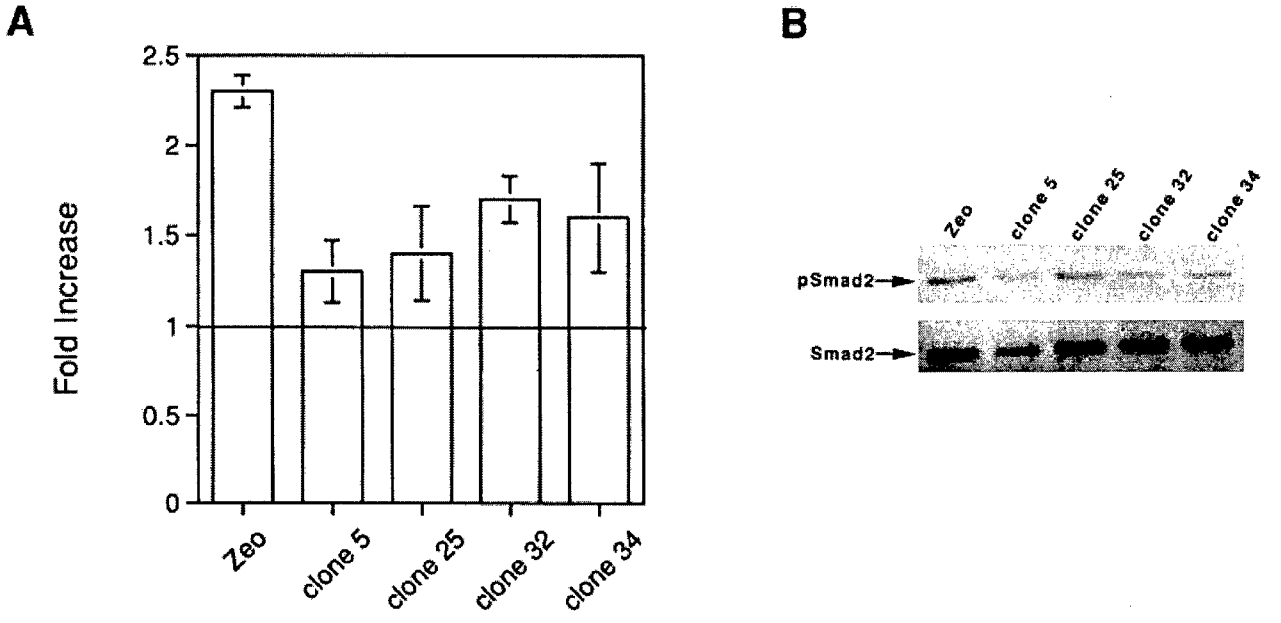

FIGURE 7 – Gene transcription in dominant negative TGF-β receptor II expressing clones. (*a*) 4T1 cells stably transfected with a mock vector (Zeo) or the dominant negative truncated TGF-β receptor II (clones 5, 25, 32 and 34) were transiently co-transfected with p3TP-lux reporter and VR1412 β-galactosidase plasmid DNA. Cells were then treated with rhTGF-β1 for an additional 18 hr before determination of luciferase activity. Bars represent fold increase and standard error of triplicate samples. Luciferase activities were normalized based on β-galactosidase expression. The data shown are representative of 3 experiments. (b) 4T1 cells stably transfected with a mock vector (Zeo) or the dominant negative truncated TGF-β receptor II (clones 5, 25, 32 and 34) were treated with rhTGF-β1 and immunoblotting for Smad2 and the phosphorylated form of Smad2 was performed.

TABLE I – EXPRESSION OF TRUNCATED TGF-B RECEPTOR II SUPPRESSES METASTASES FORMATION[1]

Cell type	Tumor volume	Number of lung metastases
4T1	783.4 ± 34.2	251 ± 36
Zeo	810.7 ± 102.8	114 ± 35
clone 5	899.4 ± 110.6	10 ± 3
clone 25	606.7 ± 53.2	1 ± 1
clone 32	952.1 ± 192.4	61 ± 13
clone 34	407.2 ± 23.9	3 ± 1

[1]SCID mice were injected orthotopically with parental, mock transfected or gene-modified 4T1 cells. Three weeks post-injection, the mice were sacrificed and their lungs were collected and examined for the presence of surface metastases. Values represent the mean ± standard error (n = 4).

invade and metastasize *in vivo*. The results also exhibit that 4T1 cells are resistant to TGF-β-mediated growth inhibition yet respond to TGF-β as demonstrated by the presence of membrane receptors that bind ligand, initiate downstream phosphorylation of Smad2 and induce Smad-dependent transcriptional activation. Thus, unlike numerous cancer cells whose resistance to TGF-β growth inhibition is due to inactivation of TGF-β receptors[29–31] or Smad family signal transducers,[32] 4T1 cells are capable of TGF-β-receptor-mediated signal transduction. These findings are in concordance with those of others that have identified functional receptors in TGF-β growth resistant cancers such as glioma[33], melanoma[34] and cervical carcinoma.[35] The intactness of Smad-mediated gene transcription in the 4T1 mammary tumor cell line is similar to that reported in human melanoma cells in which Smad-mediated gene transcription was shown to be independent of cell proliferation.[34]

The TGF-β-induced morphological changes that we observed in 4T1 mammary tumor cells are consistent with those reported by others in transformed murine mammary epithelial cells,[15] murine colon cancer cells[14] and skin carcinoma cells[16] and have been correlated with metastatic potential.[14–16] Recent work by Piek et al.[18] suggests that Smad proteins may play a role in the invasive and metastatic phenotype. Using the NMuMG murine mammary epithelial cell line they demonstrated the requirement for Smad2 and Smad4 proteins in inducing transdifferentiation in cells constitutively expressing low levels of type I receptor (ALK-5). The transition from the epithelial to the mesenchymal phenotype was characterized by stress fiber formation and downregulation/relocalization of E-cadherin, changes frequently associated with the invasive and metastatic phenotype[14,15]. Similar Smad-dependent pathways present in 4T1 tumor cells may contribute to their invasive and metastatic activities. That 4T1 cells secrete TGF-β and exhibit basal levels of Smad2 phosphorylation suggests a cell autonomous mechanism of TGF-β signaling which may contribute to the metastatic phenotype as has been proposed in murine models of renal, lung, mammary and colon cancers.[14,17,19] Experiments are in progress to resolve this question.

The importance of TGF-β in the invasion and metastasis of 4T1 tumor cells was demonstrated in studies in which expression of a truncated dominant negative receptor significantly suppressed formation of lung metastases *in vivo*. Whereas metastasis formation was severely curbed, tumorigenicity at the primary tumor injection site was not affected. This observation is in agreement with that of Yin et al.[19] using the human breast cancer cell line MDA-MB-231 in which they exhibited that dominant-negative blockade of the type II TGF-β receptor had no effect on the growth of local tumor yet significantly diminished metastasis to the bone.[19] In our study, histological analysis did not reveal any discernible differences in the morphology or local invasiveness of the primary tumors between mock-transfected (4T1-Zeo) and DNRII-expressing tumors. In addition, no histologic evidence of hematogenous metastases was observed in blood vessels adjacent to the primary tumors. These findings are not unexpected since hematogenous invasion of neoplastic cells is a relatively infrequent occurrence that is rarely documented histologically. A more reliable indicator of hematogenous invasion of carcinoma cells is the presence of metastases at

distant sites such as the lung. The decreased number of lung metastases in the DNRII group is therefore considered a significant finding in our study and reflective of decreased hematogenous invasion of the carcinoma cells that express the mutant receptor. Taken together, our findings suggest that TGF-β may preferentially exert its effect on cellular genes that control invasion and spread of tumor cells.

Our observation that the DNRII affected *in vivo* metastasis but *not in vitro* migration and invasion suggests that *in vitro* assays are limited in their ability to predict metastatic capacity and that characteristics other than increased motility are required for spread to occur *in vivo*. TGF-β may promote metastasis in part through its ability to regulate the expression or activity of extracellular matrix-degrading proteases like MMP-9 whose role in tumor metastasis is well established.[36,37] Our finding that TGF-β upregulates the secretion of MMP-9 by 4T1 cells (unpublished observation) is in agreement with this mechanism. Understanding the TGF-β-induced downstream effectors mediating the metastatic phenotype will likely lead to the identification of molecular targets that once perturbed could override this phenotype.

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Autocrine Transforming Growth Factor-β Signaling in Mammary Tumor Cell Invasiveness. Nancy Dumont and Carlos L. Arteaga, Departments of Cell Biology and Medicine, Vanderbilt University School of Medicine and Vanderbilt-Ingram Cancer Center.

Transforming growth factor-β (TGFβ) is a pleiotropic growth factor, which plays a critical role in modulating cell growth, differentiation and plasticity. There is increasing evidence that after cells lose their sensitivity to TGFβ-mediated growth inhibition, autocrine TGFβ signaling may potentially promote tumor progression. Studies have shown that introduction of a dominant negative TGFβ type II receptor (TβRII) in a variety of tumor cells can delay tumor formation and prevent the conversion of cells from an epithelial to a more invasive and metastatic mesenchymal phenotype. The purpose of this study is to identify the biochemical pathways perturbed by a dominant negative TβRII that are causally associated with the metastatic phenotype of tumors. In order to do that, the MDA-MB-231 human breast cancer cell line was stably transfected with either a kinase-dead TβRII-K277R (dnTβRII) construct or the vector control. Affinity labeling of cell surface receptors with ^{125}I-TGFβ1 revealed an increase in the labeling of TβRII on the surface of cells transfected with dnTβRII compared to the vector control or parental cells, suggesting that the dnTβRII was indeed expressed. This was confirmed by immunoprecipitating the affinity-labeled exogenous receptor via its HA tag. The function of the transgene was evaluated by i) examining phosphorylation and nuclear translocation of Smad2, a TGFβ signal transducer, and ii) measuring transcription utilizing the TGFβ responsive promoters, p3TP-lux and p(CAGA)$_{12}$-lux. These assays revealed that TGFβ-mediated Smad2 phosphorylation, Smad2 nuclear translocation, and transcriptional responses were reduced in cells stably transfected with dnTβRII compared to vector control. In addition, wound closure assays indicated that the basal migratory potential of dnTβRII expressing cells was impaired. Biochemical experiments are currently underway to identify the signal transducers perturbed by the dnTβRII expression which impair motility, and which may, in turn, be relevant to TGFβ-mediated invasion and metastases.

ABSTRACT GORDON CONFERENCE

Expression of a Kinase Inactive TGFβ Type II Receptor Impairs Motility in Breast Cancer Cells

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Transforming growth factor-β (TGFβ) is a pleiotropic growth factor, which plays a critical role in modulating cell growth, differentiation and plasticity. There is increasing evidence that after cells lose their sensitivity to TGFβ-mediated growth inhibition, autocrine TGFβ signaling may potentially promote tumor progression. Studies have shown that introduction of a dominant negative TGFβ type II receptor (TβRII) in a variety of tumor cells can delay tumor formation and prevent the conversion of cells from an epithelial to a more invasive and metastatic mesenchymal phenotype. The purpose of this study is to identify the biochemical pathways perturbed by a dominant negative TβRII that are causally associated with the metastatic phenotype of tumors. To accomplish this aim, the MDA-MB-231 metastatic human breast cancer cell line was stably transfected with either a kinase-dead TβRII-K277R (dnTβRII) construct or the vector control. Affinity labeling of cell surface receptors with ^{125}I-TGFβ1 revealed an increase in the labeling of TβRII on the surface of cells transfected with dnTβRII compared to the vector control or parental cells, suggesting that the dnTβRII was expressed. This was confirmed by immunoprecipitating the affinity-labeled exogenous receptor via its HA tag. The function of the transgene was evaluated by i) examining phosphorylation and nuclear translocation of Smad2, a TGFβ signal transducer, and ii) measuring transcription utilizing the TGFβ responsive promoters, p3TP-lux and p(CAGA)$_{12}$-lux. These assays revealed that TGFβ-mediated Smad2 phosphorylation, Smad2 nuclear translocation, and transcriptional responses were reduced in cells stably transfected with dnTβRII compared to vector control. In addition, wound closure assays indicated that the basal migratory potential of dnTβRII expressing cells was impaired, independent of changes in proliferation. This impairment in motility appears to be TGFβ specific in that it can be restored by expression of a constitutively active type I TGFβ receptor, but not by constitutively active activin or BMP type I receptors. Biochemical experiments are currently underway to identify the signal transducers perturbed by dnTβRII expression which impair motility, and which may, in turn, be relevant to TGFβ-mediated invasion and metastases.